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NOVEL INSIGHTS INTO DISHEVELLED AND FRIZZLED FUNCTION

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Cover: stylized graphics of the intracellular part of Frizzled interacting with the heterotrimeric G protein.

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“Show me a completely smooth operation and I'll show you someone who's covering mistakes. Real boats rock.”

~ Frank Herbert, from Dune

ABSTRACT

The formation of a multicellular body during embryonic development is one of the most fascinating processes in biology. The cells are in constant communication and must be precisely coordinated. Several essential signalling pathways have been identified in cell-to-cell signal transmission. One such pathway is the WNT signalling network, which is highly conserved and plays an important role not only in embryogenesis but also in the maintenance of adult tissue homeostasis. Disturbances of the WNT signalling system are connected to many pathophysiological processes including cancer, rendering this pathway a great target for pharmacological treatment.

The aim of this thesis is to broaden our knowledge regarding the function of the two main components of the WNT signalling cascade: the intracellular scaffold protein Dishevelled (DVL) and the membrane receptors from the Class Frizzled (Class F).

First, we have shown that DVL is located at the centrosome. We have explored the function of DVL in the regulation of the centrosomal cycle – a chain of events that centrosome undergoes during the cell cycle progression. DVL constitutes a part of the linker connecting two centrosomes that coordinate centrosomal separation. As a target of several kinases, DVL possesses many phosphorylation sites, which regulate its function and selectivity for its binding partners. We added another kinase to DVL's portfolio of kinases – the centrosomal kinase Nima related kinase 2 (NEK2). Interaction between NEK2 and DVL is important for the regulation of the centrosomal cycle, where phosphorylation of DVL by NEK2 displaces DVL from the centrosome together with other linker proteins subsequently enabling centrosomal separation. Therefore, DVL represents a newly identified key player in the centrosomal cycle.

Next, we investigated the ability of receptors from the Class F to signal through heterotrimeric G proteins. We have shown that Frizzled 10 (FZD_{10}) forms an inactive state complex with one specific isoform of heterotrimeric G proteins, G13, and this complex dissociates after addition of ligands for FZD_{10}. Additionally, FZD_{10} overexpression induced Yes-associated protein/Tafazzin (YAP/TAZ) transcriptional activity. We have also found that FZD_{10} mRNA is expressed in endothelial cells of the developing brain. Since G13 is a crucial molecule in vascular development, particularly for the endothelial cells, we propose that FZD_{10} signalling through G13

and YAP/TAZ might represent a novel signalling axis in the development of the central nervous system (CNS) vasculature.

Further, we explored the structure and function of another FZD homologue, FZD_6. We identified a triad of conserved cysteine residues in the linker region, which is connecting the N-terminal cysteine rich domain (CRD) with the core of the receptor. These cysteines are crucial for the localization of FZD_6 in the plasma membrane as well as for its interaction with the cytoplasmic scaffold protein DVL.

Additionally, we utilized a method involving receptor purification and insertion into artificial phospholipid bilayer particles to investigate the ability of FZD_6 to directly activate heterotrimeric G proteins. We showed that FZD_6 activates Gi as a monomeric unit by its constitutive activity. The method itself harbours great potential for future biochemical and pharmacological studies.

Taken together, this thesis provides new evidence for the involvement of the WNT pathway components in the less investigated WNT signalling events, connects DVL biology with the centrosomal cycle regulation, expands the portfolio of FZD homologues forming an inactive preassembly complex with heterotrimeric G proteins, and presents FZDs as direct activators of heterotrimeric G proteins.

LIST OF SCIENTIFIC PUBLICATIONS

INCLUDED IN THE THESIS:

1. **Dishevelled is a NEK2 kinase substrate controlling dynamics of centrosomal linker proteins.**
   Igor Cervenka, **Jana Valnohova**, Ondrej Bernatik, Jakub Harnos, Matej Radsetoulal, Katerina Sedova, Katerina Hanakova, David Potesil, Miroslava Sedlackova, Alena Salasova, Zachary Steinhart, Stephane Angers, Gunnar Schulte, Ales Hampl, Zbynek Zdrahal and Vitezslav Bryja.
   Proc Natl Acad Sci U S A. 2016 Aug; 113(33):9304-9, DOI: 10.1073/pnas.1608783113

2. **FZD_{10}-$G\alpha_{13}$ signalling axis points to a role of FZD10 in CNS angiogenesis.**
   Belma Hot, **Jana Valnohova**, Elisa Arthofer, Katharina Simon, Jaekyung Shin, Mathias Uhlén, Evi Kostenis, Jan Mulder and Gunnar Schulte.
   Cell Signal. 2017 Apr; 32:93-103; DOI: 10.1016/j.cellsig.2017.01.023

3. **Functional dissection of the N-terminal extracellular domains of Frizzled 6 reveals their roles for receptor localization and Dishevelled recruitment.**
   Jana Valnohova, Maria Kowalski-Jahn, Roger K. Sunahara and Gunnar Schulte.
   J Biol Chem. 2018 Sep. IN PRESS. DOI: 10.1074/jbc.RA118.004763

4. **FZD_6 acts as a guanine nucleotide exchange factor for heterotrimeric Gi1 proteins in a reconstituted system.**
   Jana Valnohova, Prahnesh A. Venkataraman, Julian Petersen, Roger K. Sunahara and Gunnar Schulte.
   (MANUSCRIPT)

NOT INCLUDED IN THE THESIS:

1. **Dishevelled enables casein kinase 1–mediated phosphorylation of Frizzled 6 required for cell membrane localization.**
   Katerina Strakova, Maria Kowalski-Jahn, Tomas Gybel, **Jana Valnohova**, Vishnu M. Dhople, Jakub Harnos, Ondrej Bernatik, Zbynek Zdrahal, Jan Mulder, Cecilia Lindskog, Vitezslav Bryja, Gunnar Schulte. J Biol Chem. 2018 Oct. IN PRESS.

CONTENT

LIST OF ABBREVIATIONS

AHD	α-helical domain
APC	Adenomatous polyposis coli
ABC	ATP binding cassette transporter family
Apo AI	Apolipoprotein AI
BBB	Blood-brain barrier
BRB	Blood-retina barrier
CaMK	Calmodulin-dependent protein kinase
CDK1	Cyclin-dependent kinase 1
CDK5RAP2	CDK5 regulatory subunit-associated protein 2
Celsr	Cadherin EGF LAG seven-pass G-type receptor 1
CEP135	Centrosomal protein of 135 kDa
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate
CK1	Casein kinase 1
CK2	Casein kinase 2
Class F	Class Frizzled
C-NAP1	Centrosomal Nek2-Associated Protein 1
CNS	Central nervous system
co-IP	Co-immunoprecipitation
CRD	Cysteine rich domain
Cryo EM	Cryo electron microscopy
DAG	Diacylglycerol
DAPLE	DVL-associating protein with a high frequency of leucine residues
dcFRAP	Dual/double colour fluorescent recovery after photobleaching
DDM	n-dodecyl β-D-maltoside
DEP	Dishevelled, Eg-10 and Pleckstrin
DIX	Dishevelled and Axin domain
DMR	Dynamic mass redistribution
DRY	Asp-Arg-Tyr motif (in Class A GPCR)
DSH	Dishevelled (in *Drosophila melanogaster*)
DVL	Dishevelled
E	Embryonic day
ECD	Extracellular domain
ECL	Extracellular loop

Eg5	Kinesin related motor protein Eg5
ERK 1/2	Extracellular signal-regulated kinase 1/2
FEVR	Familial exudative vitreoretinopathy
FRAP	Fluorescent recovery after photobleaching
FRET	Förster/fluorescence resonance energy transfer
FZD	Frizzled
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GLUT1	Glucose transporter-1
GNTI$^-$	N-acetylglucosaminyltransferase I activity deficient HEK cells
GPCR	G protein-coupled receptor
GSK-3β	Glycogen synthase kinase-3β
GTP	Guanosine triphosphate
HDL	High-density lipoprotein particle
HEK293	Human embryonal kidney 293 cells
ICL	Intracellular loop
IMAC	Immobilized metal-ion affinity chromatography
int1	*Integration1*
IP_3	Inositol triphosphate
JNK	c-Jun-N-terminal kinase
LRP5/6	Low-density lipoprotein receptor-related protein 5/6
MMTV	Mouse mammary tumour virus
MNG	Lauryl maltose neopentyl glycol
MSP	Membrane scaffold protein
MTOC	Microtubule organizing center
mTOR	Mammalian target of rapamycin
NEK2	Nima related kinase 2
NLK	NEMO-like kinase
NMR	Nuclear magnetic resonance spectroscopy
PAR-1	Serine/threonine protein kinase par-1
PCP	Planar cell polarity
PDZ	PSD-95/Disc large/ZO-1 homologous
PECAM-1	Platelet endothelial cell adhesion molecule-1
PIP_2	Phosphoinositol diphosphate
PLC	Phospholipase C
PLK1	Polo-like kinase 1
PLK4	Polo-like kinase 4

PNVP	Perineural vascular plexus
PTM	Post-translational modification
PTX	*Bordetella pertussis* toxin
R	Receptor in resting state
R*	Receptor in activated state
RGS	Regulators of G protein signalling
rHDL	Reconstituted high-density lipoprotein particle
ROI	Region of interest
ROR2	Receptor tyrosine kinase-like orphan receptor 2
RYK	Receptor-like tyrosine kinase
S2 cells	Schneider 2 cells
SAS-6	Spindle assembly abnormal protein 6 homologue
sFRP	Secreted FZD-Related Protein
siRNA	Small interfering ribonucleic acid
SMO	Smoothened
SPR	Surface plasmon resonance
STIL	SCL-interrupting locus protein
TcdB	*Clostridium difficle* toxin B
TCF/LEF	T-cell factor/lymphocyte enhancer factor
TM	Transmembrane
TSPAN12	Tetraspanin 12
Vangl	Van Gogh-like protein
VEGF-A	Vascular endothelial growth factor A
VFD	Venus flytrap domain
wg	Wingless
WNT	Wingless/*integration1*
YAP/TAZ	Yes-associated protein/Tafazzin
γ-TuRC	γ-tubulin ring complexe
ΔCRD	Construct lacking the N-terminal CRD domain

1 INTRODUCTION

1.1 WNT signalling pathways

Communication among cells in multicellular organisms is extremely complex and involves many pathways. These pathways are not usually linear signalling events and they are subject to many regulatory mechanisms with positive and negative feedback loops and multiple cross-talks to other signalling networks to efficiently coordinate the sophisticated developmental processes. One group of these pathways is regulated by proteins of the WNT family. The WNT signalling network can be historically divided into two main signalling branches. The WNT/β-catenin pathway, also referred to as 'canonical' is the most intensively studied and also the best investigated, which is partially caused by the availability of well-defined readouts of the pathway activity. The other branch, WNT/β-catenin-independent signalling, sometimes called 'non-canonical', represents a whole network of signalling pathways, which are not yet well understood. Both branches of WNT signalling appear to antagonize each other; however, the underlying mechanisms remain to be elucidated (Ishitani et al., 2003; Torres et al., 1996).

WNT signalling research started with the identification of two genes. In 1973, a mutant of *Drosophila melanogaster* lacking wings was described and named Wingless (wg) (Sharma, 1973). In 1982, a mouse mammary tumour virus (MMTV) was reported to induce mammary gland tumours by activating a gene called *integration1* (*int1*) (Nusse and Varmus, 1982). A few years later it was shown that these two genes are homologues (Rijsewijk et al., 1987) and the name became a *portmanteau* of Wingless and *integration1* – WNT (Nusse et al., 1991).

1.1.1 WNT/β-catenin signalling

The WNT/β-catenin pathway garnered much attention because of its role in the regeneration of the intestinal epithelium (Gregorieff and Clevers, 2005). It was discovered, that this pathway regulates proliferation and self-renewal of the intestinal stem cells. When impaired, it represents the main driving force for development of colorectal cancer (Phelps et al., 2009). More generally, this pathway is necessary for the maintenance of various tissues in addition to intestine, such as hair follicles or bones (Veltri et al., 2017). Not only homeostasis of adult tissues, but also embryonic

development is critically dependent on the balanced activity of the pathway. In the larval development of *Drosophila*, the WNT/β-catenin pathway controls polarity of body segments, where naked belts (high concentration of WNT) alternate with denticles (low concentration of WNT) (Bejsovec and Martinez Arias, 1991). In vertebrates, it is involved in the establishment of the primary body axis and anterior-to-posterior orientation of the embryo (Hikasa and Sokol, 2013). Ectopic expression of the WNT-1 mRNA in the ventral blastomeres of the embryo results in body axis duplication (McMahon and Moon, 1989).

The pathway uses β-catenin as the main signal transduction molecule. The transcriptional regulator β-catenin is present in several pools in the cell, mostly in adherent junctions and cytoskeleton, and a minor pool in the cytoplasm (Valenta et al., 2012). It is the cytoplasmic pool of β-catenin that WNT signalling uses for signalling purposes. Interestingly, it has been reported that the pools of β-catenin are

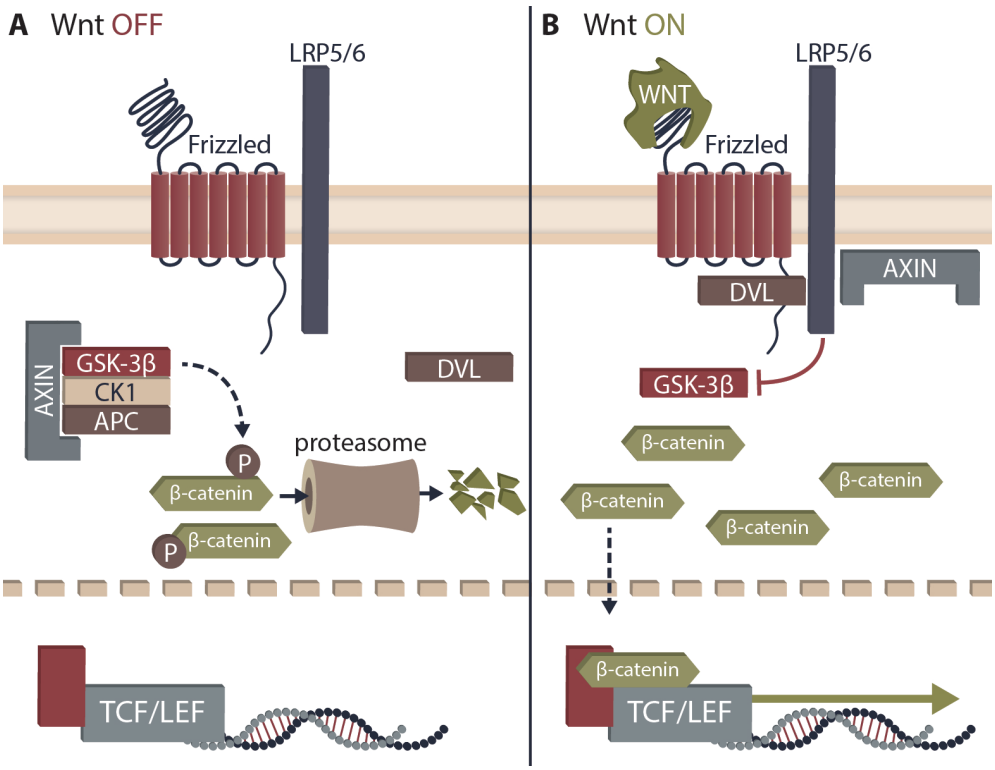

Figure 1. The WNT/β-catenin pathway. (A) In the absence of WNTs, the pathway is inactive, β-catenin is phosphorylated and targeted for proteasomal degradation. (B) In the presence of WNTs, the destruction complex is disabled, β-catenin accumulates and translocates into the nucleus, where it regulates transcription of TCF/LEF transcription factor-regulated WNT target genes.

not completely isolated and cytoplasmic β-catenin can be released from or bound to the adherent junctions (Heasman et al., 1994; Maretzky et al., 2005). This opens many possibilities of alternative modulation of the WNT/β-catenin signalling pathway through extracellular cues that relate to substrate recognition or cell shape. However, the regulation of these events remains obscured.

In the absence of WNTs, when the pathway is inactive, the levels of β-catenin in the cytoplasm are kept low (**Figure 1**). β-catenin is continuously phosphorylated by the destruction complex, which consists of the scaffold proteins AXIN (Behrens et al., 1998), Adenomatous polyposis coli (APC) (Munemitsu et al., 1995), Casein kinase 1 (CK1) (Amit et al., 2002) and Glycogen synthase kinase-3β (GSK-3β) (Rubinfeld et al., 1996). This phosphorylation marks β-catenin for the recruitment of an E3 ubiquitin ligase and subsequent proteasomal degradation (Hart et al., 1999; Kitagawa et al., 1999).

Activation of the WNT/β-catenin pathway is triggered by the presence of WNTs, which bind to the receptor complex consisting of FZD and low-density lipoprotein receptor-related protein 5/6 (LRP5/6). The cytoplasmic scaffold protein DVL is then translocated to the plasma membrane where it interacts with FZD (Klingensmith et al., 1994; Tauriello et al., 2012). AXIN in complex with GSK-3β is also sequestered to the plasma membrane by the interaction of the DIX domains of DVL and AXIN. The intracellular tail of LRP5/6 is phosphorylated by the kinases GSK-3β (Tamai et al., 2004) and CK1 (Davidson et al., 2005), which subsequently leads to the inhibition of GSK-3β activity (Cselenyi et al., 2008; Stamos et al., 2014). GSK-3β cannot phosphorylate β-catenin anymore, therefore β-catenin accumulates in the cytoplasm and translocates to the nucleus. There, it acts as a transcriptional regulator in concert with the T-cell factor/lymphocyte enhancer factor (TCF/LEF) (Brunner et al., 1997; Molenaar et al., 1996) inducing expression of genes such as *cyclin D* (Shtutman et al., 1999), *c-myc* (He et al., 1998) and *axin-2* (Jho et al., 2002).

1.1.2 WNT/β-catenin-independent signalling

The most recognised signalling pathways within the β-catenin-independent signalling cascade influence planar cell polarity (WNT/PCP pathway) and levels of intracellular calcium (WNT/Ca^{2+} pathway). In comparison to the β-catenin-dependent pathway, these pathways modulate cell motility, migration and the

polarisation of tissues (Humphries and Mlodzik, 2018). They also regulate gene transcription, but the genes are independent from TCF/LEF transcription factors.

There are several other pathways that are assigned to the network of β-catenin-independent WNT pathways: WNT/Receptor tyrosine kinase-like orphan receptor 2 (ROR2), WNT/Receptor-like tyrosine kinase (RYK), WNT/Mammalian target of rapamycin (mTOR) and WNT/Protein kinase A (PKA), but their signalling mechanisms are poorly understood (Schulte, 2010; Semenov et al., 2007; Sherwood et al., 2014).

1.1.2.1 The WNT/PCP pathway

PCP signalling regulates several important processes such as morphogenesis and tissue patterning. Most typically, it establishes polarity within the plane of the epithelium. The prototype tissue used to study planar cell polarity is *Drosophila* wing, because it develops from a flat single-layer epithelium (Maung and Jenny, 2011). However, processes employing the same conserved mechanisms were also described in vertebrates. For example, during convergent extension, PCP provides directional input leading to tissue remodelling resulting in elongation of the anterior-to-posterior axis (Jones and Chen, 2007). PCP also influences hair patterning and was characterised as the crucial pathway for the positioning of stereocilia in the inner ear (Jones and Chen, 2008).

The main outcome of the PCP pathway activity is the establishment of the asymmetric localization of the core PCP molecules at the opposite sides of the cell

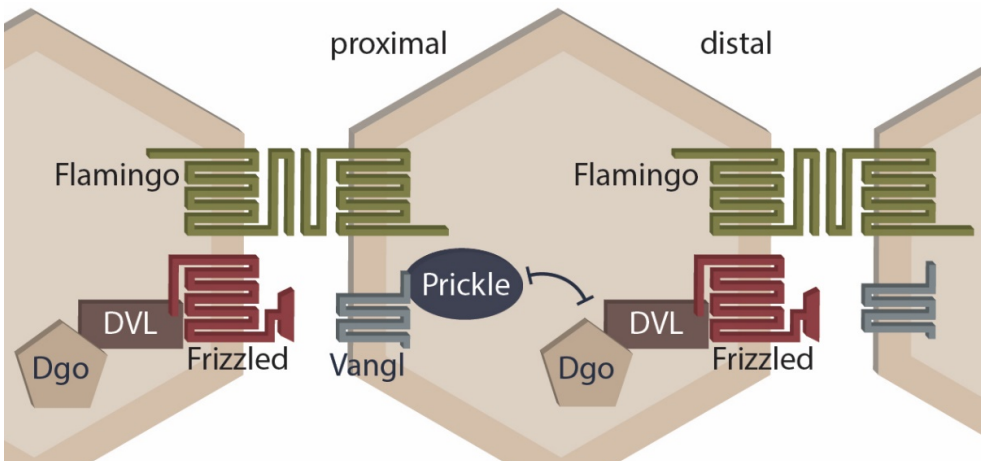

Figure 2. Schematic overview of the PCP components and their organization in epithelial cells (modified from Devenport, 2014)

(**Figure 2**) (Peng and Axelrod, 2012). The molecular complex formed on the proximal side of the cell consists of Van Gogh-like protein (Vangl) and Prickle, while the so-called distal subset includes FZD, DVL and Diego/Inversin. The complexes are connected between the cells via a cadherin-like protein, Flamingo/Cadherin EGF LAG seven-pass G-type receptor 1 (Celsr), present on both sides of the cell (Usui et al., 1999). Each of the protein subsets regulates signalling events engaged in both positive and negative feedback loops to establish the tissue polarity (Strutt and Strutt, 2008).

Activation of this pathway results in signalling through small GTPases, RAC1 (Habas et al., 2003), RHO A (Wünnenberg-Stapleton et al., 1999) and CDC42 (Djiane et al., 2000) and typically leads to cytoskeletal changes. The components of the PCP pathway are described to be upregulated in some types of cancer and given the fact that the pathway control cell movements it is not surprising that it promotes the invasiveness of cancer cells (Luga et al., 2012).

1.1.2.2 The WNT/Ca^{2+} pathway

WNT signals can also increase the intracellular levels of calcium. The typical calcium response triggered by G protein-coupled receptor (GPCR) activation is mediated by heterotrimeric G proteins. G proteins then activate phospholipase C (PLC), which triggers the production of diacylglycerol (DAG) and inositol triphosphate (IP_3) from phosphoinositol diphosphate (PIP_2). IP_3 stimulates calcium channels on the membrane of the endoplasmic reticulum, resulting in the release of calcium ions (Ca^{2+}). Ca^{2+} in turn activates calcium-dependent protein kinases, such as PKC, or binds to the regulatory protein calmodulin, which modulates Calmodulin-dependent protein kinases (CaMK) (Heldin et al., 2016).

The involvement of WNTs in calcium signalling was first demonstrated in *Danio rerio*, in which calcium release was observed after overexpression of WNT-5A (Slusarski et al., 1997a). Calcium fluxes control the activity of multiple kinases. The activation of some of them has been also reported after WNT stimulation. WNT-5A and FZD_2 were for example shown to promote the translocation of PKC to the plasma membrane (Sheldahl et al., 1999), whereas WNT-5A and WNT-11 were shown to activate CaMKII (Kühl et al., 2000).

The WNT/Ca^{2+} pathway regulates ventral cell specification, where it antagonises WNT/β-catenin signalling (Kühl et al., 2000). Defective calcium

signalling results in the dorsalization of the embryo, effect similar to overactive β-catenin signalling (Kühl et al., 2000; Westfall et al., 2003).

It has been suggested that the WNT/Ca^{2+} and WNT/PCP signalling might overlap to a certain degree. First, both of them use DVL for signal transduction (Sheldahl et al., 2003). Second, the overexpression of Prickle, a core component of the WNT/PCP pathway, also mediates calcium flux (Veeman et al., 2003).

1.2 Selected components of the WNT signalling pathways

1.2.1 Frizzleds

The *frizzled* gene was described for the first time in *Drosophila*, where its mutations resulted in the perturbed polarity of bristles on wings, thorax and legs (Vinson and Adler, 1987; Vinson et al., 1989). It was only several years later that FZD was characterised as a receptor for WNT proteins (Bhanot et al., 1996). The proof of WNT-FZD interaction was provided by work done in Schneider 2 (S2) cells, which do not respond to WNT by increased β-catenin stabilization, most likely because they do not express FZDs. Introducing FZD into these cells by transfection enabled WNTs to bind to the cell surface and activate the WNT/β-catenin pathway. After the human genome sequencing project was finished, ten homologues of the *frizzled* gene (FZD_{1-10}) were discovered in humans and mammals and the phylogenetic analysis revealed their close relationship to Smoothened (SMO), a GPCR involved in Hedgehog signalling (Schulte, 2010).

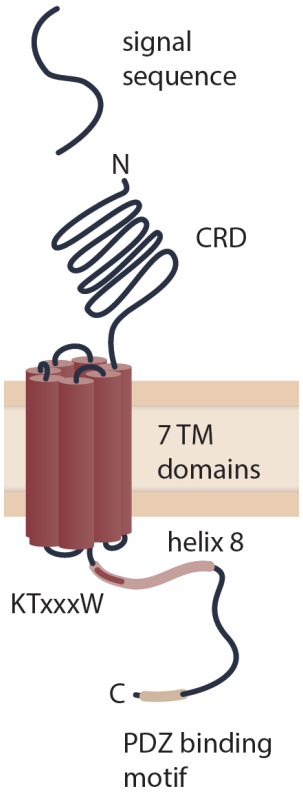

Figure 3. Schematic representation of the FZD structure.

FZDs consist of one polypeptide chain that spans the plasma membrane seven times (**Figure 3**), an architecture typical for membrane proteins of the GPCR superfamily. Although architecturally similar to classical GPCRs, FZDs lack some of the typical domains, which led to their separate classification as

receptors in the Class Frizzled (Class F). At the N-terminus, FZDs have a cleavable signal sequence, which ensures its insertion into the plasma membrane. After that, a large and highly conserved cysteine rich domain (CRD) follows, which consists of roughly 120 amino acids. Since the CRD contains five disulphide bonds connecting α-helical stretches and β-sheets, it is considered to be structurally rigid (Dann et al., 2001). This domain is also thought to be the main interaction site for WNTs, although some reports describe it to be dispensable for WNT binding (Chen et al., 2004). The transmembrane part consists of seven helices and forms three extracellular (ECL) and three intracellular loops (ICL). The ECLs contain conserved cysteines creating disulphide bonds, which are typical for other GPCRs where they contribute to receptor stabilization (Wang et al., 2006a). Moreover, they might play a role in ligand recognition or binding, as in other GPCRs (Lee et al., 2015). The ICLs take part in binding downstream components, of which interaction with DVL is one of the best described (Gammons et al., 2016; Strakova et al., 2017; Tauriello et al., 2012; Umbhauer et al., 2000). Although FZDs do not possess typical motifs for G protein binding that are conserved in Class A GPCRs, such as the Asp-Arg-Tyr (DRY) motif at the transmembrane domain (TM) 3 or NPxxxY at TM7, there are charged residues in the ICL3, which are known to be essential for G protein coupling in other GPCRs (Schulte, 2010). Right after TM7, a completely conserved KTxxxW motif has been identified in all FZD isoforms. This motif binds the PSD-95/Disc large/ZO-1 homologous (PDZ) domain of DVL (Wong et al., 2003) and is essential for the activation of the WNT/β-catenin pathway (Umbhauer et al., 2000). Almost all FZDs possess another PDZ binding motif at the very end of the C-terminus, which is implicated in binding other cytoplasmic proteins (Wang et al., 2006a). Predictions showed that some of the FZD homologues can create an α-helical stretch at the C-terminus (Gayen et al., 2013), which has been confirmed in recent crystal structure of FZD_4 (Yang et al., 2018). This helical structure, called helix 8, is also present in other GPCRs and was suggested as a determinant for G protein coupling (Wess et al., 2008).

Since aberrant WNT signalling is involved in many pathological processes, FZDs represent a promising target for pharmacological intervention. However, the development of any drugs and especially small molecules, which would modulate the receptor activity, has been shown to be very challenging (Schulte and Wright, 2018). The biggest challenge is to produce ligands, which would have selective specificity

just for one FZD homologue. FZD homologues themselves are quite different not only structurally but also functionally, the modelling with the purpose of drug design was just partially successful due to the lack of a reliable assays for direct screening of the receptor activation and lack of the knowledge about the ligand binding mechanism (Schulte and Wright, 2018; Zeng et al., 2018; Zhang et al., 2017). So far, drug screens mainly relied on the dual-luciferase reporter assay for detection of the WNT/β-catenin signalling activity, called TOPFlash (Korinek et al., 1997), where the possibility for detecting off-target effects is high and the likelihood to uncover FZD specific drug is low.

Progress in the drug design can be accelerated by solving the structure of FZD. Until recently, high resolution structural information has only been available for several CRD domains, including CRD of FZD_8 in absence (Dann et al., 2001) or presence (Janda et al., 2012) of the WNT protein. FZD models of the TM core have been based on its close relative member of Class F, SMO, which has been crystalized with several small molecule ligands (Byrne et al., 2016; Huang et al., 2018; Wang et al., 2013, 2014). However, the crystal structure of the FZD_4 transmembrane part was just recently solved in the ligand free state (Yang et al., 2018). The study discusses possible reasons for the limited progress in the design and binding of small molecules ligands to the TM core of FZDs. In the FZD_4 homologue, the binding pocket in the receptor core seems to be too narrow and possibly too hydrophilic, which could partially explain the failed attempts at modulating FZD activity. Understanding the mechanism of ligand binding, having the reliable screening assay for receptor activation and insight into the receptor structure including CRD would greatly speed-up the development of ligands or small molecules, which could be used as a medical treatment of WNT and FZD related diseases.

1.2.1.1 FZD_6

The largest FZD, FZD_6, consists of more than 700 amino acids and is classified in the same homology cluster as FZD_3 (Schulte, 2010). These two homologues share higher sequence similarity, and both of them function exclusively in the WNT/β-catenin-independent pathways, typically in the WNT/PCP pathway.

FZD_6 regulates processes equivalent to the patterning of *Drosophila* cuticle, such as hair patterning in mice (Guo et al., 2004; Wang et al., 2006b) and together with FZD_3 redundantly controls neural tube closure, axon growth and guidance and the planar orientation of hair bundles in the inner ear (Dong et al., 2018; Hua et al.,

2014; Wang et al., 2006c). The WNT/β-catenin-dependent and -independent pathway act mostly antagonistically (Ishitani et al., 2003; Torres et al., 1996), probably by using some of the common components. In agreement with that, FZD_6 serves as a negative regulator of the WNT/β-catenin pathway, probably by activating NEMO-like kinase (NLK), which inhibits WNT/β-catenin signalling (Golan et al., 2004).

Some studies suggest that FZD_6 acts as a tumour suppressor by inhibiting the tumorigenic β-catenin pathway (Han et al., 2018; Yan et al., 2016). FZD_6 has been implicated in the repression of gastric cancer (Yan et al., 2016). Gastric cancer cells exhibited decreased levels of FZD_6 and overexpression of FZD_6 in these cells led to inhibition of cell proliferation, and surprisingly, also cell migration. A similar effect was observed in the case of prostate cancer. Flavonoid luteolin inhibited WNT/β-catenin signalling by upregulation of FZD_6, which suppressed stemness of prostate cancer cells (Han et al., 2018). On the other hand, FZD_6 is sometimes associated with increased invasion and metastatic potential of cancer cells due to its ability to polarize the cells. FZD_6 is frequently amplified in breast cancers where it was shown to regulate motility and invasion (Corda et al., 2017). Moreover, FZD_6 has been highly expressed in liver tumours (Chen, 2018), colorectal cancer (Kim et al., 2015) and chronic lymphocytic leukaemia where it is involved in their malignant progression (Wu et al., 2011). Expression of FZD_6 also predicts poor prognosis for patients with neuroblastoma (Cantilena et al., 2011) and glioblastoma (Hirano et al., 2014). As mentioned above, FZD_6 seems to be involved in invasion and migration, properties connected to PCP pathway outcomes, rather than proliferation.

The most typical human disease associated with FZD_6 mutations is nail dysplasia. This rare recessive disorder manifests in nail malformations, which are occurring in highly inbred families. Several mutations in FZD_6 have been associated with this disease: Glu584X and Arg511Cys (Fröjmark et al., 2011; Naz et al., 2012), Gly559Asp (Kasparis et al., 2016), Ser620Cys (Mohammadi-asl et al., 2017) and Gly422Asp (Raza et al., 2013). These mutations are either missense mutations or causing a frameshift resulting in truncated products and proteins with most likely impaired in membrane localization (Fröjmark et al., 2011). Further investigation of FZD_6 in the claw and nail development showed that it regulates expression of the genes connected to differentiation of epidermis (Cui et al., 2013).

Some aspects of FZD_6 activation and signalling have been described by our group. FZD_6 forms an inactive state complex with Gi and Gq but not Go, Gs and G12

(Kilander et al., 2014a). The FZD-G protein 'preassembly' is lost by uncoupling the FZD_6-Gi complex with PTX, with agonist stimulation (WNT-5A) and seems to be regulated by DVL. We also showed that FZD_6 is able to dimerize (Petersen et al., 2017). The dimer dissociates upon WNT-5A stimulation resulting in increase of the monomer population. The monomeric receptors promote signalling through phosphorylated extracellular signal-regulated kinases 1/2 (ERK1/2). Dimer dissociation upon agonist stimulation is transient and the receptors re-associate in a similar time frame as ERK1/2 signalling is attenuated.

FZD_6 represents the prototype homologue for PCP signalling and even though it is not able to signal via β-catenin, understanding its signalling is of importance. Moreover, targeting of FZD_6 in cancers might be favourable, since it will avoid the side effects caused by the disrupted β-catenin signalling.

1.2.1.2 FZD_{10}

Although FZD_{10} belongs to the same homology cluster as FZD_9 and FZD_4, one of the most investigated FZD homologue, the research of FZD_{10} is lagging behind and gained attention primarily because of its upregulation in synovial sarcomas (Fukukawa et al., 2008; Nagayama et al., 2005). With the exception of placenta, FZD_{10} has not been detected in any of the adult tissues (Moriwaki et al., 2000) and its expression in most commonly used cell lines is extremely low. Expression of FZD_{10} seems to be rather limited to embryonic development, especially in neural tissues and limb buds, where it is associated with the WNT/β-catenin pathway activity (Galli et al., 2014; Garcia-Morales et al., 2009; Kawakami et al., 2000).

FZD_{10} is highly upregulated in synovial sarcomas (Nagayama et al., 2002). This rare disease is a type of soft tissue sarcoma, which occurs in limbs, often in close proximity to the joints. Almost all synovial sarcomas are associated with a translocation t(X,11), resulting in a fusion protein acting as a transcriptional regulator (Lee et al., 1993)(Brett et al., 1997). The role of FZD_{10} in the development or progression of this disease is not clear, but signalling through the DVL-Rac1-JNK (c-Jun-N-terminal kinase) pathway points to an involvement of the WNT/β-catenin-independent pathway (Fukukawa et al., 2009). Except for the upregulation in synovial sarcomas, FZD_{10} was reported to be involved also in other types of cancer, such as colorectal cancer (Nagayama et al., 2009; Scavo et al., 2018) and breast cancer (Gong et al., 2014).

Since FZD_{10} is not expressed in normal adult tissues, it seems to be an attractive target for a therapy, where antibodies against FZD_{10} are labelled with different radioligands in order to selectively kill the synovial sarcoma tumorigenic cells (Fukukawa et al., 2008; Giraudet et al., 2018; Li et al., 2018).

1.2.2 Dishevelled

The best described direct interaction partner for FZDs is the scaffold protein DVL. It plays an equally crucial role in both WNT/β-catenin-dependent and -independent signalling pathways as a scaffold protein and a signal transducer (Gao and Chen, 2010). It is speculated that DVL serves as a switch or relay among these pathways, but how is the signalling propagated to the downstream components and understanding of its ability to distinguish among different signalling branches remains a mystery (Mlodzik, 2016).

DVL was discovered in *Drosophila melanogaster* and was named after a phenotype, in which the orientation of hairs and bristles is disrupted (Fahmy and Fahmy, 1959; Klingensmith et al., 1994). *Drosophila* has only one isoform of DVL (in *Drosophila* marked as DSH) compared to mammals that have three, referred to as DVL1, DVL2 and DVL3. These isoforms can functionally compensate for each other to some extent (Mlodzik, 2016; Wynshaw-Boris, 2012).

Binding of WNT to FZDs results in phosphorylation of some of the WNT signalling components, including DVL. Phosphorylated DVL translocates to the plasma membrane, where it sequesters AXIN, a protein of the destruction complex. AXIN cannot serve anymore as a negative regulator in this complex, β-catenin is not targeted for degradation and triggers transcription of the WNT target genes.

DVL contains three globular domains, the N-terminal DIX domain, the central PDZ domain and the Dishevelled, Eg-10 and Pleckstrin (DEP) domain followed by a C-terminal region (**Figure 4**) (Gao and Chen, 2010). DIX domain is associated with the ability of DVL to polymerize via a head-to-tail interaction, which

Figure 4. Schematic representation of the DVL structure. DVL contains three structured domains, DIX, PDZ and DEP, which are interconnected by unstructured regions.

is also the main mechanism of sequestering AXIN from the destruction complex (Schwarz-Romond et al., 2007a). The centrally located PDZ domain serves as an interface platform between DVL and its binding partners and was shown to directly interact with the KTxxxW motif present at the C-terminus of FZDs (Wong et al., 2003). The DEP domain is assumed to interact with membrane lipids (Simons et al., 2009) and might be responsible for membrane localization of DVL (Pan et al., 2004). Recently, the mechanism of the DEP function was described in more detail (Gammons et al., 2016; Paclíková et al., 2017). The DEP domain is responsible for DVL dimer formation via so-called 'domain swapping' between two DVL molecules that subsequently triggers its polymerization dependent on the DIX domain. The C-terminus of DVL possesses a regulatory function. It can be highly phosphorylated and is associated with autoinhibition of DVL activity (Bernatik et al., 2011). Additionally, there are two other highly conserved regions (Penton et al., 2002). The first one, a basic region, contains conserved serine and threonine residues and is located between the DIX and PDZ domains. Proline-rich region is located between the PDZ and DEP domains. They play a role in protein-protein interactions and are probably involved in channelling of signals between WNT/β-catenin-dependent and -independent signalling.

Originally, it was hypothesized that DVL interacts with FZD through the KTxxxW motif at the C-terminus of the receptor (Umbhauer et al., 2000; Wong et al., 2003), but additional FZD interfaces for DVL interaction were discovered. DVL binds motifs in the ICL3 (Tauriello et al., 2012), but DVL also interacts with a quite large intracellular surface on the FZD, including all three ICLs (Gammons et al., 2016).

Since DVL can bind such a large number of proteins, it is not clear how it redirects the signals among distinct branches. It might be enabled by interaction with its binding partners but also through posttranslational modifications. The most intensively studied modification is phosphorylation, which is a highly dynamic process. The identified kinases phosphorylating DVL are for example CK1 (Klimowski et al., 2006; Peters et al., 1999), CK2 (Willert et al., 1997), PAR-1 (Bernatik et al., 2011; Sun et al., 2001) and Nima related kinase 2 (NEK2) (Schertel et al., 2013).

1.2.3 FZD ligands

For some time, scientists have been puzzled as to which ligands bind FZDs. This puzzle has been solved in 1996 and FZDs wre identified as receptors for lipoglycoproteins from the WNT family (Bhanot et al., 1996). WNTs act as morphogens and they are required in embryonic development (Nusse, 2003; Struhl and Basler, 1993). However, WNTs are also crucial regulatory molecules in adulthood, where WNT proteins regulate stem-cell renewal and tissue homeostasis (Pinto et al., 2003).

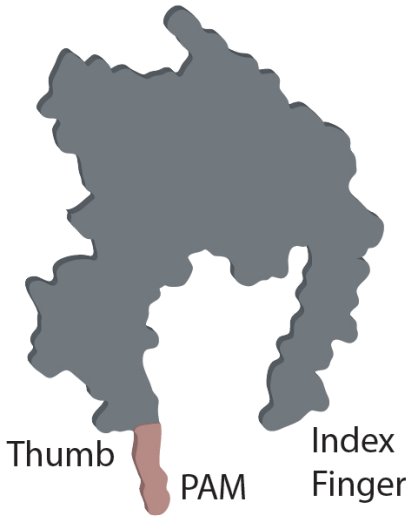

Figure 5. Scheme of the WNT protein. The structure possesses two domains: the 'thumb' and the 'index finger' domain, which are seen as the receptor contact points. PAM – palmitoleic acid (modified from Janda et al. 2012).

Homologues of WNTs are evolutionary conserved within the animal kingdom, and 19 different WNTs have been identified in mammals (Wodarz and Nusse, 1998). The gene *Wnt* was first discovered in *Drosophila melanogaster* as a mutation causing absence of wing and halters (Sharma and Chopra, 1976). WNTs are 350 to 400 amino acids large proteins, containing 22 conserved cysteine residues involved in formation of disulphide bonds (Janda et al., 2012; Willert and Nusse, 2012). Such a cysteine network is sometimes referred to as a 'cysteine knot'. Solving the crystal structure of *Xenopus* WNT-8 together with CRD of FZD_8 determined the architecture to be a two domain structure: a larger, N-terminal domain, resembles a 'thumb', and a smaller C-terminal domain is designated as an 'index finger' (**Figure 5**) (Janda et al., 2012). A palmitoleic acid modification was identified at the tip of the 'thumb', at Ser187 in *Xenopus* WNT-8 and probably interacts with FZD's CRD. Another interaction site of WNT with the CRD was described at the tip of the 'index finger'.

WNTs carry several posttranslational modifications such as acylation and glycosylation (Willert and Nusse, 2012). The function of N-glycosylation in WNT signalling and secretion is not clear and the number of glycosylated sites varies for different homologues (Doubravska et al., 2011; Komekado et al., 2007; Kurayoshi et

al., 2007; Mason et al., 1992). On the other hand, acylation is essential for the WNT activity, but the number of lipidations or the exact type of the modifications are still a matter of debate. Palmitate was assumed to be essential for signalling (Kurayoshi et al., 2007; Schulte et al., 2005; Willert et al., 2003) and palmitoleic acid for secretion (Takada et al., 2006). Later it was shown that signalling by WNT proteins only requires palmitoleic acid modification and that it is necessary to have at least one lipidation for secretion, regardless of its type (Tang et al., 2012). The acetyltransferase Porcupine catalyses the addition of the lipid (Tanaka et al., 2000) and only such modified WNT can associate with a transporter protein called Wntless (also called Evi or Sprinter), which escorts WNT to the plasma membrane (Bänziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006). Recent studies suggest the role of the lipid moiety in dimerization of FZD CRDs (DeBruine et al., 2017), where the fatty acid occupies a large hydrophobic pocket created by the CRD dimers (Nile et al., 2017). Until the key discovery of the lipid modification on WNT molecules, it has been impossible to purify biologically active WNTs (Schulte et al., 2005; Willert et al., 2003). Addition of relatively high concentrations of detergents (particularly CHAPS) during the extraction process overcame the challenge. In general, WNT proteins seem to be relatively unstable. Importantly, tagging WNTs also decreases their stability and therefore careful placement of the tag and monitoring WNT activity is required (Schulte et al., 2005). An additional modulation of WNT activity is mediated by trans-membrane WNT-specific metalloproteases, TraB domain containing-protein 2A and 2B, known as Tiki1 and Tiki2 respectively, that cleave the N-terminus of WNT proteins leading to WNT inactivation (Zhang et al., 2012, 2016).

WNTs can have either a short or long operational range. For short range, WNTs are probably still associated to the membrane of the secreted cell and can act on the same or the neighbouring cell (Farin et al., 2016). WNTs can also travel on filopodia-like protrusions, called cytonemes (Holzer et al., 2012). For long range, the lipid moiety needs to be shielded in the aqueous solution. Several proteins possessing the transporter role have been discovered. The protein Swim facilitates the extracellular diffusion of WNT in the *Drosophila* wing disc (Mulligan et al., 2012). Similarly, an albumin-like serum component, Afamin, associates with WNT-3A secreted into the culture media (Mihara et al., 2016). Interestingly, secreted FZD-Related Proteins (sFRP), which are considered to sequester WNTs and therefore

inhibit WNT signalling, also appear to be able to transport them (Bovolenta et al., 2008; Mii and Taira, 2009). For long range transport, WNTs can also be inserted into lipid containing membranous particles such as exosomes (Luga et al., 2012), high- and low-density lipoproteins (Neumann et al., 2009) and vesicles called argosomes (Greco et al., 2001). Moreover, WNTs associate with extracellular matrix components especially to glycosaminoglycans (Bradley and Brown, 1990; Reichsman et al., 1996), which maintain their solubility and support their ability to signal (Lin, 2004).

The 19 different WNTs can bind to ten different FZD homologues and selectivity of their interaction still remains to be elucidated. It is also not completely clear how cells can respond to specific WNT expression patterns of multiple WNTs, which sometimes have opposing biological effects. One theory is that FZDs require a specific co-receptor, which would confer the specificity for each WNT. Such an example can be FZD_4 interaction with Norrin enabled by LRP5/6 and Tetraspanin 12 (TSPAN12) during retina vascularization (Lai et al., 2017).

It was surmised that WNTs are the only extracellular ligands that bind FZDs, but over time other proteins have been identified as FZD ligands. For example, sFRPs belong to a family of secreted modulators of the WNT signalling pathway. Although, they are considered to be mainly WNT binding molecules, it has been shown that they also serve as ligands for FZDs (Üren et al., 2000). Another ligand, Norrin, is specific just for FZD_4 (Xu et al., 2004). Although it contains a cysteine knot motif, Norrin is not related to the WNTs. Norrin was identified to cause the Norrie disease, an eye disorder caused by impaired retinal development. Mice with a mutation in FZD_4 exhibited phenotypes similar to mice with a Norrin mutation (Berger at all 1996). The accessory protein TSPAN12 has been reported as a component of the Norrin-FZD_4 complex and directly associates with Norrin-FZD_4 maximal signalling capacity (Lai et al., 2017). Recent findings pointed out that FZD_1, FZD_2 and FZD_7 can bind the toxin B (TcdB) produced by *Clostridium difficle* (Tao et al., 2016). Association of TcdB was shown to compete for binding with WNTs and can block the WNT mediated signalling through β-catenin.

1.3 The centrosome

Eukaryotic cells possess many organelles to ensure its proper functionality. Some of them are indispensable, some of them seem to be dispensable in certain cell

types or organisms. The centrosome belongs to these mysterious organelles. It is present in almost all metazoan cells apart from oocytes (Szollosi et al., 1972). Interestingly, plants also lack centrosomes but they have developed alternative mechanisms taking over the centrosomal functions (Schmit, 2002).

Centrosomes can perform a variety of tasks in different cell types during the progression of the cell cycle. One of the main functions is anchoring and nucleating of microtubules hence also the name microtubule organizing center (MTOC) (Wu and Akhmanova, 2017). The microtubules are in charge of transporting cargo within the cell to maintain the cell shape. Thus, the centrosome acts as a crucial determinant in these processes. Similarly, two centrosomes regulate the assembly of the bipolar mitotic spindle during mitosis. Since the centrosomes do not only influence the formation of kinetochore microtubules, but also the orientation of the mitotic spindle itself, it has a role in asymmetric cell division and cell fate determination (Yamashita, 2009). Besides the mitotic spindle assembly, centrosomes contribute to the progression of the cell cycle by concentrating signalling molecules and regulators of cell division (Schatten, 2008). However, the centrosome has a function also in cells, which do not undergo cell division. In these quiescent cells the centrosome can associate with the plasma membrane and transform into the basal body giving rise to cilia or flagella functioning in cellular motility and as signal transduction hubs (Marshall, 2008).

Depletion of centrosomes has different effects, depending on the stage when they were lost. *Drosophila* embryos that completely lack centrosomes die after a few cell divisions (Basto et al., 2006). If the centrosomes are depleted later within the embryonic development, the embryos are able to go through the whole embryogenesis, with just slight delay. However, the flies die after birth due to the lack of cilia. Cilia function as chemo- and mechanosensors thus movements of cilia-deficient flies are uncoordinated, and so they cannot feed. Similarly to *Drosophila*, mouse embryos lacking the centrosomes are very small and the development arrests at embryonic day 9 (E9) accompanied by dramatic apoptosis (Bazzi and Anderson, 2014).

The centrosome consists of pericentriolar material assembled around two centrioles (Bornens, 2012). The two centrioles, denoted as a mother and daughter centriole, are positioned perpendicularly to each other. When the daughter centriole starts to form, the first assembled structure is a nine-fold symmetric structure, a structure of a cartwheel, composed of proteins spindle assembly abnormal protein 6 homologue (SAS-6), centrosomal protein of 135 kDa (CEP135) and SCL-

interrupting locus protein (STIL) (Kitagawa et al., 2011). During the centriole growth, this structure is elongated through the deposition of centriolar microtubules around the cartwheel. The centrioles accumulate pericentriolar material, layers of proteins including CDK5 regulatory subunit-associated protein 2 (CDK5RAP2) and pericentrin, which recruit and activate γ-tubulin ring complexes (γ-TuRC) anchoring and nucleating microtubules (Zimmerman et al., 2004).

Like the genome, the centrosome also duplicates once per cell cycle (**Figure 6**) (Bettencourt-Dias and Glover, 2007). The cells in the G1 stage contain just one centrosome with two centrioles connected by a protein called cohesin. During the S phase, the centrioles separate and duplicate. Entering G2, the cells harbor two centrosomes, each containing two centrioles. These two centrosomes are transported to the opposite ends of the cell during mitosis and each of them becomes segregated into the newly formed cells making it possible to enter a new cell cycle.

In order to trigger the nucleation of new centrioles, and thus the duplication of the centrosome, the centrioles must separate in the first phase of the cell cycle, the G1 phase. The mother centriole is partially disconnected from the daughter by cleavage of cohesin connecting the centrioles (Schöckel et al., 2011; Tsou et al.,

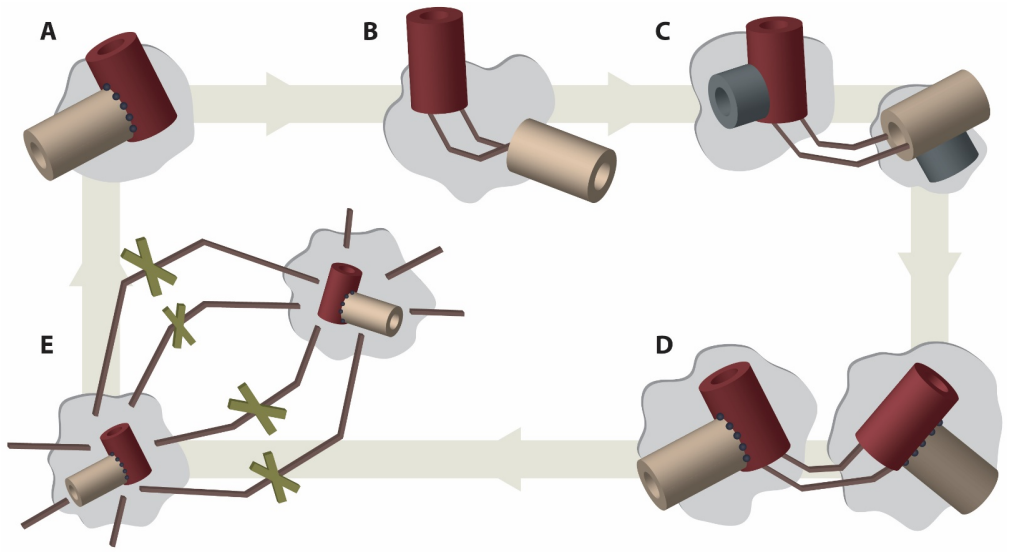

Figure 6. Schematic presentation of the centrosomal cycle. A – G1 stage of the cell cycle when the cells possess just one centrosome with two centrioles. B – separation and C – duplication of centrioles during the S phase of the cell cycle. D – duplicated centrosome connected by the protein linker in G2 phase. E – disengaged centrosomes in the mitosis (inspired by Mardin and Schiebel, 2012).

2009). Cleavage is enabled by the proteolytic activity of separase and modulated by polo-like kinase 1 (PLK1) (Tsou et al., 2009). The separation is required for the next phase, the nucleation and elongation of daughter centrioles, which are regulated by polo-like kinase 4 (PLK4). Each of the separated centrioles give rise to one new, perpendicularly attached daughter centriole. After the centrioles are duplicated, the centrosomes can be disengaged. The centrioles were separated in a previous step by cleavage of cohesin, but they are still connected by a 'loose linker' (Bornens et al., 1987) consisting of proteins including Centrosomal Nek2-Associated Protein 1 (C-NAP1), Rootletin, and the kinase NEK2. The proximal ends of the centrioles are decorated with C-NAP1 and NEK2 (Fry et al., 1998), which serve as anchor points for Rootletin that physically connects both centrioles (Bahe et al., 2005). In the G2 phase, NEK2 phosphorylates C-NAP1 (Fry et al., 1998) and Rootletin (Bahe et al., 2005), which triggers their removal from the centrosome. After disengagement, the centrosomes are physically separated by the action of the kinesin motor Eg5 (Blangy et al., 1995) that is mediated by the Aurora A - PLK1 kinase cascade. PLK1 not only stimulates separase for the cohesin removal in one of the previous steps, but it also regulates activity of NEK2 and triggers action of kinesin related motor protein Eg5 together with Cyclin dependent kinase 1 (CDK1) (Mardin and Schiebel, 2012).

Since the discovery of the centrosome by Flemming and Boveri, it was surmised that centrosomal aberrations are associated with tumorigenesis (Godinho and Pellman, 2014). Indeed, multipolar mitoses are often observed in tumour cells. An aberrant number of centrosomes leads to chromosome instability, and thus promotes the tumorigenesis (Basto et al., 2008). Individuals with defects in centrosomes suffer from developmental disorders affecting the brain function such as primary autosomal recessive microcephaly (Nigg et al., 2014) or the entire body resulting in dwarfism (Klingseisen and Jackson, 2011). Additionally, several diseases broadly classified as ciliopathies are associated with malfunction of the centrosome-associated structure, the primary cilium (Fliegauf et al., 2007).

The importance of the centrosome in coordination and regulation of the cell cycle and implication of the centrosome in aneuploidy and tumorigenesis are considered as important targets for anti-cancer drugs (Venghateri et al., 2015). Several compounds targeting the centrosomal proteins, exclusively kinases, have been developed. They are still in preclinical phase or early stages of clinical trials. However, they represent a promising avenue to achieve tumour suppression.

1.4 Angiogenesis in the CNS

The formation of new blood vessels occurs via two mechanisms. *De novo* formation of blood vessels during embryogenesis, where endothelial precursors (angioblasts) migrate and differentiate, is called vasculogenesis. In the second type of the blood vessels formation, angiogenesis, endothelial cells sprout from the pre-existing vessels.

Vascularization of the CNS is mediated entirely by angiogenesis. It starts at E8.5 - E9.5 when angioblasts differentiate and form a network of vessels called perineural vascular plexus (PNVP) around the neural tube. Around E9.5, the sprouting from the PNVP invades the neuroepithelium following the proangiogenic signals such as Vascular endothelial growth factor A (VEGF-A). The CNS has the highest requirements for oxygen and nutrient supply and it is also very sensitive to toxic agents. To prevent CNS damage, the endothelial cells establish a special protective layer, called blood-brain barrier (BBB).

The BBB has very strict requirements for a well-organized system of tight junctions between the endothelial cells. Many molecules cooperate to prevent the free flow of substances between CNS and blood, including claudins, occludins, junctional adhesion molecules and endothelial selective adhesion molecules (Dejana 2004). The mature BBB capillaries are further covered by a high number of pericytes embedded in a common basal membrane together with astrocytic end-feet that are indispensable for regulation of the selective BBB permeability (Armulik et al., 2010). There is an obvious need for a vivid communication between neural tissue and the circulation. Therefore, there are many specialized molecules in the endothelial cells of the BBB that enable transport of substances, such as ATP binding cassette transporter family (ABC) or glucose transporter-1 (GLUT1) that are considered to be one of the earliest BBB markers (Liebner and Plate, 2010). It is not exactly clear, when the BBB is established during embryonic development. Initially it was thought that it is formed after invasion and remodelling of vessels to the CNS. However, recent reports argue that angiogenesis and establishment of the BBB happen simultaneously (Umans et al., 2017).

The profound understanding of the underlying mechanisms of BBB function is of utmost interest, since BBB dysfunctions are associated with human diseases such as stroke, brain tumours and multiple sclerosis. The investigation of BBB function in more detail will enable a more effective treatment of patients affected by BBB defects.

1.5 G protein-coupled receptors and heterotrimeric G proteins

GPCRs are the largest class of membrane proteins encoded in the human genome. They play a central role in many physiological processes including sense of vision, taste and olfaction, cell survival, proliferation, differentiation, chemotaxis and cell-cell communication (Hall et al., 1999; Pierce et al., 2002). The diseases connected to impaired function of GPCRs range from obesity, heart failure, asthma, schizophrenia to cancer.

GPCR represent a suitable drug targets due to their involvement in a large number of (patho)physiological processes as well as their cell surface localization. The feasibility of GPCRs for medical intervention is highlighted by the fact that up to 60% of prescribed pharmaceuticals target GPCRs directly or indirectly (Overington et al., 2006; Rask-Andersen et al., 2011). The world-best selling drugs includes Advair Diskus for asthma treatment, a drug combining steroids with long acting β_2 adrenoreceptor agonists, and Abilify, a partial agonist for several GPCRs, for treatment of schizophrenia.

Activation of GPCRs can be triggered by various extracellular ligands such as photons, ions, nucleotides, amines, odorants, lipids, peptides and proteins (Wacker

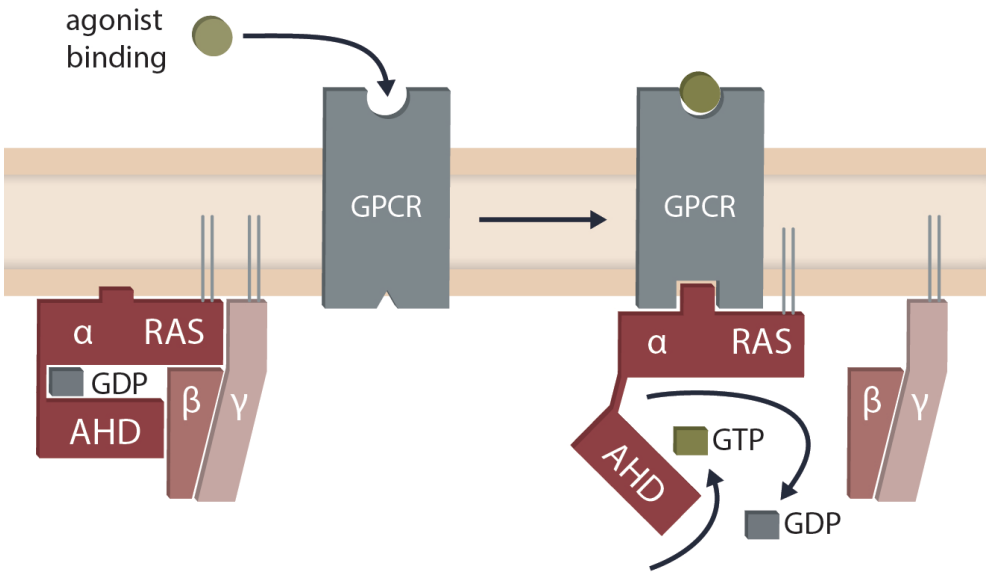

Figure 7. The initial process of G protein activation by GPCR. After agonist binding, the receptor establishes a ternary complex with G protein. The GDP bound between the RAS and AHD is released and exchanged for GTP (modified from Rasmussen et al. 2011).

et al., 2017). In response to the signals, GPCRs undergo a conformational rearrangement, which results in activation of downstream molecules. In the classical GPCR signalling pathway, the activation translates into signalling through heterotrimeric G proteins, however, GPCRs can also interact with other cytoplasmic scaffold proteins, such as β-arrestins.

Heterotrimeric G proteins consist of three subunits, α, β and γ. The α subunit possesses two distinct domains, the RAS and α-helical domain (AHD), where the RAS domain associates with the receptor and AHD has quite large flexibility (Rasmussen et al., 2011). The guanine nucleotide binding site for GDP or GTP is located between them. The G proteins are classified in four different groups, Gs, Gi/o, Gq/11 and G12/13 (Milligan and Kostenis, 2006). Each of them is having specific signalling outcomes including regulation of adenylyl cyclase, signalling through RHO kinases or mobilization of calcium.

For heterotrimeric G proteins, GPCRs serve as guanine nucleotide exchange factor (GEFs) facilitating the exchange of GDP for GTP (**Figure 7**). The nucleotide exchange is accompanied by the structural rearrangements in both, the heterotrimeric G protein and the receptor. It results in a dissociation of the α subunit from βγ subunits and activation of the G protein. A closer look at the α subunit during activation reveals that its C-terminal α5 helix in the RAS domain is protruding into the GPCR cavity upon association with the active receptor, where it interacts with TM5 and TM6 causing an outward move of TM6 (Carpenter and Tate, 2017). The AHD moves away from the RAS domain allowing a wide opening. This opening is necessary for the release of GDP from the nucleotide binding site. However, the exposure of the nucleotide binding site alone is not enough for nucleotide release, and it is expected that other structural rearrangements, especially in the RAS domain are required for nucleotide dissociation (Dror et al., 2015). The nucleotide free state of the G protein is short-lived in cells because of the high intracellular concentration of GTP, which rapidly associates with the α subunit causing dissociation of βγ. Then, the G protein passes the signal further to effector molecules such as adenylyl cyclase and phospholipases. In order to limit the lifetime of the G protein signal, the α subunit possesses an intrinsic GTPase activity, which enables the hydrolysis of GTP back to GDP. This mechanism, together with the action of the regulators of G protein signalling (RGS), ensures reliable ‘switch off’ of the G protein and enables the re-association with the βγ subunit.

Several models were developed in order to better understand the role of G proteins for receptor activation. In the two-state model, where the G protein is not included, the receptor is presented in the resting state (R) and activated state (R*), which exist in equilibrium. Without any ligand, the equilibrium is usually strongly shifted towards R. Agonists have higher affinity for R* and thus they shift the equilibrium towards R*. The discovery of the ability of heterotrimeric G protein to act a as positive allosteric modulator, meaning that it is able to increase the binding of the agonist to the receptor, motivated the upgrade of the simple two-state model to the ternary complex model (**Figure 8**) (De Lean et al., 1980). The revised model does not only take the interaction between a ligand and its receptor but also the active receptor and G protein into account. It gives rise to a four-point 2D model – a ternary complex model, where the G protein serves as a regulatory component and establishes the high affinity agonist-receptor-G protein complex. In this state, no nucleotide is bound and is referred to as the active state. However, the regulatory component can be represented by other molecules, for example ligands binding to other than the primary (so-called orthosteric) site of the receptor, thus modulating its affinity to the orthosteric ligands. Alternatively, such a regulatory unit can be also represented by β-arrestin.

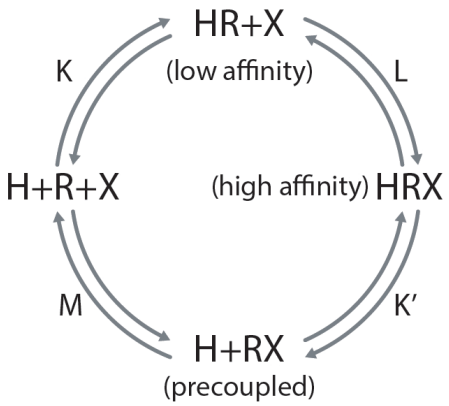

Figure 8. A ternary complex model. H – hormone (ligand), R – receptor, X – additional component (G protein). The G protein is required to establish a high affinity complex of receptor with bound ligand (HRX) (modified from DeLean et al., 1980).

Initially, β-arrestin binding to the receptor and β-arrestin-mediated endocytosis were seen as a mechanism of desensitization of GPCR signalling (therefore the name 'arrestin' depicting signalling arrest) (Lohse et al., 1990). However, except for desensitization and internalization of GPCRs, they also facilitate their own mode of signalling that can be initiated independently or right after G protein activation.

The typical structure of GPCRs presents with seven transmembrane passing helices, thus the name 7-transmembrane receptor. Since the GPCR superfamily

includes up to 800 members, they have been divided into six classes, A-F (Kolakowski, 1994). Recently, a newer classification was created to better reflect the phylogenetic relationship organizing them into five families: Glutamate, Rhodopsin, Adhesion, FZD/Taste2 and Secretin, forming the 'GRAFS' classification system (Foord et al., 2005; Schiöth and Fredriksson, 2005). Each family possesses some unique domains, structures or features. For example, the DRY motif at the TM3 or NPxxxY are typical determinants for the Rhodopsin family (Audet and Bouvier, 2012). The secretin family possesses an N-terminal extracellular domain (ECD), which binds mainly polypeptide hormones (Hollenstein et al., 2014). Representatives of the glutamate family (Class C) has a large N-terminus containing a conserved venus flytrap domain (VFD) and a CRD (Chun et al., 2012). Adhesion family receptors possess a domain, which undergoes proteolysis (Araç et al., 2012).

Crystal structures of GPCRs, especially in complex with G proteins have greatly contributed to a better understanding of their function. Rhodopsin was the first GPCR to be crystallized (Palczewski et al., 2000). It is highly enriched in the retina and thus, bovine retina was used as a natural source of this receptor for purification. However, other GPCRs are usually only present in natural sources in small quantities, which is one of the reasons why it took another seven years to obtain the crystal structure of the second GPCR, the β_2 adrenergic receptor (Rasmussen et al., 2007). Since then, around 200 structures of more than 30 distinct GPCRs have been published and they have a remarkable influence on the design of the new drugs (Pándy-Szekeres et al., 2018).

2 AIMS

The general aim of this thesis is to extend the knowledge about the WNT signalling pathway in the less conventional cellular processes and signalling events. The focus was mainly on two of the components, DVL and FZD.

The specific aims were to:

- Investigate the role of DVL in centrosome regulation
- Examine whether FZD_{10} interacts with and activates heterotrimeric G proteins
- Examine, which downstream signalling pathways are initiated by a putative FZD_{10}-G protein complex
- Dissect the role of the CRD and linker domain in FZD_6 functionality
- Investigate the ligand dependency of the FZD-DVL interaction
- Optimize FZD_6 purification and incorporation of the purified receptor into artificial phospholipid bilayer particles (rHDL)
- Investigate guanine nucleotide exchange activity of purified FZD_6 towards heterotrimeric G proteins

3 METHODS

3.1 Fluorescence recovery after photobleaching

Different methods can be used to visualize and analyze dynamic cellular processes. One such technique is the fluorescent recovery after photobleaching, FRAP. It allows to observe lateral mobility of the fluorescently tagged membrane proteins, but it can be also modified to monitor the association of two molecules.

The principle of FRAP is based on the observation of the fluorescent intensity in a small region in the cell. In this region of interest (ROI) the fluorescent molecules are irreversibly bleached by a high intensity laser beam. Subsequently, the diffusion of the non-bleached proteins from the surroundings into the bleached area leads to recovery of the fluorescence over time. The speed and extent of the fluorescence recovery depend, among other parameters, on the size and the lateral mobility of the protein of interest. If the protein is highly mobile, the bleached area will recover quickly. If only a fraction of the molecules is mobile, only partial recovery of the fluorescent signal can be measured. If the protein is immobile, no recovery is detectable.

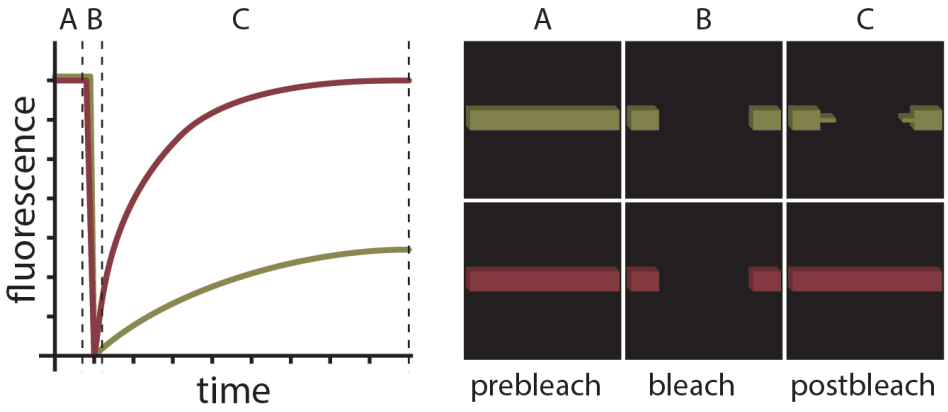

Figure 9. A schematic presentation of the dcFRAP methodology. The green molecule was immobilized (for example by crosslinking). The mobility of the red molecule is investigated. Free mobility (therefore no association) results in full recovery in the postbleach time course – represented by the scheme. (In case of association, no or partial recovery would be observed).

Monocolor FRAP was modified for observation of two colours, thus dual-colour (dc) FRAP was developed (**Figure 9**). DcFRAP can be used for the observation of association of two molecules (Picard et al., 2006). The two molecules are fluorescently labelled with different fluorophores. Typically, one of the target molecules is immobilized by crosslinking. In case of the receptor, the crosslinking can be achieved chemically by application of membrane impermeable small molecules (for example EZ-link-sulfo-NHS-LC-LC-biotin), which crosslinks the primary amines of the membrane proteins. The small area in the membrane is bleached resulting in reduced signal from both fluorophores. The crosslinked protein will not exhibit any diffusion and therefore minimal recovery of the corresponding fluorophore is detected. If the other observed molecule is associated, it will exhibit retardation in its mobility. If the two molecules do not interact, the fluorescence of the mobile protein is identical to the situation when it is expressed alone.

It should be noted that this method can detect only the state of the non-activated GPCR with the G protein, termed as preassembly state. This interaction dissociates after addition of an appropriate ligand. It is unable to detect collision coupling, where freely mobile molecules couple by random collision.

The dcFRAP method was used in **paper II** to investigate the association of FZD_{10} with heterotrimeric G proteins.

3.2 Dynamic mass redistribution

Many of the traditional GPCR assays dissect the signalling into separate pathways or signalling events, such as stimulation of the adenylyl cyclase or mobilization of calcium. However, receptor response to a stimulus can be more complex and can modulate more than one signalling event at a time. Thus, especially for the investigation of drug effects, it is advantageous to combine classical assays with approaches monitoring the overall response of the cells such as dynamic mass redistribution (DMR) technique.

DMR monitors changes in the localization (redistribution) of the biomolecules in the cells in real time. The redistribution can be caused by receptor stimulation and triggers various cellular processes such as protein trafficking, cytoskeletal rearrangements and receptor internalization. The changes are assessed in a plate reader system, where the cells are seeded on a specialized plate with waveguide-grating embedded in its bottom. The grating interacts with the cells on its

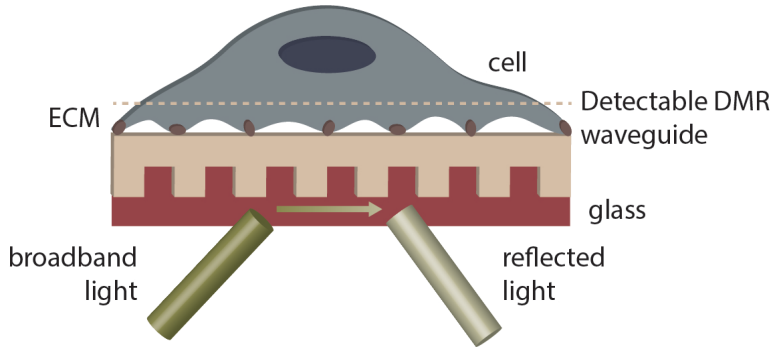

Figure 10. The principle of DMR. A cell seeded on the bottom of the DMR plate with the glass bottom containing the waveguide-grating. The bottom is illuminated with broadband light. Specific wavelength is reflected and detected (modified from Fang et al., 2007)

surface and forms an optical system. After illumination with broadband light, it can propagate and reflect a specific wavelength, which is in resonance with the system (**Figure 10**). The resonance of the optical system to a specific wavelength depends on the optical density of the cell layer. When the tested compound is added, the optical density changes as a consequence of the cellular change in shape due to the redistribution of the intracellular compounds. An increase in optical density is caused by more tight association of the cells to the surface and results in the increase of the reflected wavelength, thus producing a positive DMR signal. Similarly, a decrease in DMR reflects the reduction in optical density and decrease in the reflected wavelengths.

DMR can monitor activation of GPCRs, including activation of all G protein families (Gi/o, Gs, Gq/11 and G12/13) (Schröder et al., 2010). The DMR traces can be indicative of the specific cellular responses, because the mass redistribution is not random. In **paper II**, we employed DMR to assess signalling by FZD_{10} stimulation through G13.

3.3 Production and purification of a GPCR

Purification of membrane proteins is considered to be a challenging procedure. However, it is a necessary step for obtaining the structural information. The purified protein can be reconstituted into phospholipid bilayer particles (rHDL particles), which can be used for structure determination or for functional studies such as ligand binding assay or activation of G proteins.

Since GPCRs are generally not highly expressed in tissues, they cannot be obtained in sufficient amounts from natural sources. Perhaps, the only exception is rhodopsin, the first crystallized GPCR, which is abundant in the retina (Palczewski et al., 2000). Thus, the design of recombinant GPCRs possessing suitable purification tags in combination with a suitable production system is essential.

Several systems have been developed and used for production of suitable amounts of GPCRs. The most common expression system used for GPCR production is based on insect cells, especially the SF9 cells (*Spodoptera frugiperda*) and the HighFive cells (*Trichoplusiani*) infected with baculovirus carrying a gene for GPCR expression. Insect cells allow to produce high yields of GPCRs. In comparison to mammalian cells, the composition of the cell membranes is slightly different with low amount of cholesterol, but they possess similar posttranslational modifications. A caveat of this system can be a higher proportion of a misfolded receptor compared to mammalian systems (Thomas and Tate, 2014). Mammalian expression systems are the most suitable for receptor production because the composition of the cell membrane fits the mammalian proteins and post-translational modifications (PTMs) match the host organism. Mammalian cells have been used for both, stable and transient transfections. GNTI$^-$ human embryonal kidney (HEK) 293 cells lacking the N-acetylglucosaminyl-transferase I activity and thus lacking complex N-glycan modification have gained popularity especially for crystallization purposes. However, the mammalian cell system is also the most expensive. Alternatively, GPCRs can be expressed in yeast and bacteria, facing problems with PTMs and a different cell membrane composition.

In order to extract the receptor from the cells, the cells must be disrupted. For mammalian and insect cells, an incubation in hypotonic buffer or the disruption by nitrogen cavitation are used for membrane preparation. The nitrogen cavitation provides a better separation of the outer plasma membrane containing rather mature receptor from the internal (endoplasmic reticulum and Golgi apparatus) membranes containing also fraction of an immature receptor. After cell disruption, the receptor is solubilized in suitable detergents, which extract the transmembrane proteins from the lipid bilayer. The detergent must be efficient enough to extract the receptor from the membrane but mild enough to maintain receptor structure. The most commonly used detergent for GPCRs is n-dodecyl β-D-maltoside (DDM) (Vanaken et al., 1986) and the more recently developed lauryl maltose neopentyl glycol (MNG) (Chae et al.,

2010). MNG is more tightly associated with the receptor and provides better stability and is therefore less suitable for embedding the protein into nanodiscs, where the detergent must be stripped off the protein during reconstitution.

The protein purification procedure usually consists of several steps. The first one is typically purification with immobilized metal-ion affinity chromatography (IMAC) with either cobalt or nickel ion resins, which bind HIS tags present on the protein. Alternatively, affinity purifications are performed via FLAG or 1D4 epitope tags and corresponding antibodies. The next steps include the ion-exchange purification, based on the charge of the protein. The final purification step is often done with size exclusion chromatography, where the proteins are separated according to their size. The purification steps can be supplemented with affinity chromatography employing immobilized ligand, where the folded, ligand-binding receptors are separated from the unfolded ones. After the purification, the purity and the amount of protein are monitored by detecting of the total protein levels in a PAGE by silver or Coomassie staining.

In **paper IV**, we used different production and purification strategies, which were mostly limited by the amount of produced and bound protein. For reconstitution, usually IMAC or IMAC in combination with FLAG purification were used.

3.4 rHDL particles preparation

Once the membrane proteins are isolated and removed from their native environment, the cell membrane, they tend to unfold and aggregate. In order to extract and stabilize the membrane protein in solution, the usage of suitable detergents is important to preserve the protein structure. However, some membrane proteins still exhibit poor stability after detergent solubilization. They also might have different signalling properties (Bai et al., 2016). Moreover, the detergents could be incompatible with certain biochemical or pharmacological assays. In order to overcome these problems, membrane proteins can be further stabilized in lipid bilayers to mimic their native environment. The membrane proteins can be reconstituted into liposomes, spherical vesicles with a lipid bilayer. However, it is difficult to control for receptor orientation, stoichiometry and the size of liposomes. These problems were overcome by insertion of membrane proteins into planar discoid lipid particles. These particles, denoted as reconstituted high-density lipoprotein

(rHDL) particles or sometimes nanodiscs, come with the advantages for the investigation of membrane proteins including GPCRs (Jonas, 1986).

The structure of rHDL particles is based on physiologically occurring HDL particles in the human body. The native HDL particles are present in the plasma and blood and they participate in cholesterol homeostasis. They collect the excess of cholesterol from the periphery and transport it back to the liver for disposal (Rothblat and Phillips, 2010). HDL particles consist of phospholipids, the amphipathic protein Apolipoprotein AI (Apo AI) and the cargo cholesterol. They can either exist in nascent discoidal form or in the mature spherical form, which is predominant in the plasma.

The rHDL particles prepared *in vitro* are small discoidal particles of about 10 nm in size (Timothy H. Bayburt et al., 2002). They consist of synthetic phospholipids, which are surrounded by two molecules of the membrane scaffold protein (MSP) (Li et al., 2006; Timothy H. Bayburt et al., 2002). The MSP is the genetically engineered Apo AI, where the N-terminal globular domain, which does not interact with the phospholipids, was removed. The removal results in particles with more uniform size (Denisov et al., 2004). In the past, molecules such as ion channels (Autzen et al., 2018; Matthies et al., 2018), receptor tyrosine kinases (Mi et al., 2008), the P450 reductase (Bayburt et al., 1998) and also several GPCRs, from which the first was the β_2 adrenergic receptor (Leitz et al., 2006), were successfully embedded in rHDL.

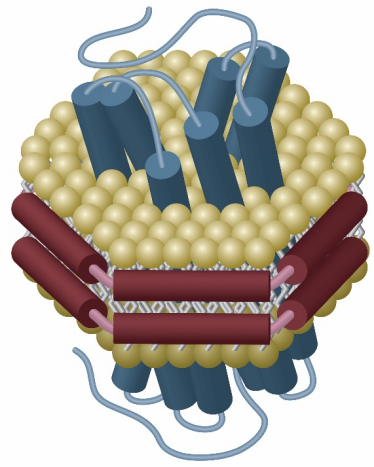

Figure 11. Idealized picture of a rHDL particle with embedded receptor (in blue). Phospholipids (in yellow) are surrounded by the two MSP/Apo AI molecules (red).

There are several ways how to 'reconstitute' (embed) the membrane protein into the rHDL particles. Membranes containing the protein of interest are solubilized in the detergent in presence of phospholipids and MSP. Obviously, this will produce particles carrying also other membrane proteins. Therefore, a subsequent purification of the target protein in the rHDL might be necessary. Another way to produce rHDL particles carrying membrane proteins is to purify the protein in a detergent, prior to the insertion into the particles. The particles

will be formed by self-assembly when the detergent is removed. The removal is usually done by dialysis or by adsorption of the detergent to porous hydrophobic beads (also termed as Bio-Beads). The target membrane protein simultaneously assembles with phospholipids into a discoidal bilayer (**Figure 11**). The size of the particles is controlled by the length of MSP. During the reconstitution, an important parameter is the lipid to MSP ratio. Too little lipids will result in deformed particles with poor lipid content. In contrast, too much lipids can form larger particles or occasionally particles containing more than two MSP proteins per particle (Brouillette et al., 1984).

The nanodisc keeps the membrane protein in solution in absence of a detergent, provides a native-like phospholipid bilayer environment and ensures membrane protein stability and functionality. The lateral diffusion of proteins is limited by the size of the MSP. Moreover, the extracellular and the intracellular sides of the protein are accessible and also allows for the control of the oligomeric state of the target membrane protein. The rHDL particles have been used for many biochemical assays, such as surface plasmon resonance (SPR), radioactive ligand binding, G protein activation assay [^{35}S]GTPγS, single molecule spectroscopy and mass spectroscopy (Bayburt and Sligar, 2010). Their biggest potential is most likely hidden in the determination of the structure of the proteins incorporated in the particle. In the past, the structure of proteins embedded in HDL particles was determined by nuclear magnetic resonance spectroscopy (NMR) (Kijac et al., 2007). However, rHDL were also successfully used for structure determination via Cryo electron microscopy (Cryo EM) (Matthies et al., 2018).

The preparation of rHDL particles is a key method for **paper IV**. Purified FZD_6 was reconstituted into rHDL particles with and without heterotrimeric G protein in order to quantify the receptor's GEF activity using a [^{35}S]GTPγS binding assay.

3.5 [^{35}S]GTPγS assay

Many functional assays for GPCRs depend primarily on the measurement of G protein-mediated generation of the second messengers such as cAMP or inositolphosphate or downstream signalling such as calcium mobilization or kinase activation. However, measurement of events proximal to the receptor activation can be advantageous, because the signal is not influenced by the additional downstream

amplification or regulation. One of the earliest processes following GPCR stimulation is a heterotrimeric G protein activation, which can be measured by the [^{35}S]GTPγS assay.

GPCRs form a ternary complex consisting of its ligand occupying the extracellular part of the receptor and a G protein binding from the intracellular side. After agonist stimulation, the activated GPCR facilitates the exchange of guanidine diphosphate (GDP) for guanidine triphosphate (GTP) on the α-subunit of the heterotrimeric G protein. This in turns leads to conformational changes in both receptor and heterotrimeric G proteins, which result in dissociation of the α-subunit from βγ and mediation of the signal to other downstream components.

The [^{35}S]GTPγS assay measures the exchange of GDP to GTP facilitated by GPCR activation. The signal is based on the non-hydrolysable analogue of GTP, where the γ oxygen in GTP is substituted by a radioactively labelled sulphur [^{35}S]. This modification renders GTP resistance to hydrolysis by the GTPase activity of the α-subunit, so it stays bound to the receptor.

The assay is more suitable for GPCRs coupled to Gi/o because of the high exchange rate of GDP for GTP but has also been used for other types of heterotrimeric G proteins (Milligan, 2003). In the classical format of the assay, the bound [^{35}S]GTPγS is measured after separation from free nucleotides by filtration on filter plates. The radioactivity captured on a filter plate is quantified by scintillation after addition of the scintillation cocktail.

We employed the measurement of G protein activation for the receptor embedded in the rHDL particles in **paper IV**.

4 RESULTS AND DISCUSSION

4.1 WNT signalling in centrosomal events

(Paper I)

WNT signalling is known to promote the cell cycle through stimulation of gene expression of *cyclin D* and *c-myc*, which enables the cells to pass the G1/S checkpoint. However, it now seems that WNT signalling can regulate the cell cycle not only by transcriptional activation, but also by the direct association with centrosomal proteins and proteins playing role in the cell division.

Over the years, it has been observed that many components of the WNT pathway change expression during the cell cycle progression. Many of them, such as β-catenin (Olmeda et al., 2003), AXIN (Kim et al., 2009), AXIN2 (Hadjihannas et al., 2012), DVL (Kikuchi et al., 2010) and APC (Dikovskaya et al., 2004), peak in the G2/M phase during the cell cycle. The main focus of the study was DVL biology, so we performed the cell sorting based on the cell cycle phases and stained individual fractions with a DVL3 antibody. We observed that DVL reaches the peak in the G2/M phase. Interestingly, it has been reported that a response of the FZD co-receptor LRP6 to WNTs is maximal also during the G2/M phase (Davidson et al., 2009). In G2/M, LRP6 undergoes a priming phosphorylation via a Cyclin Y/CDK14 complex increasing its sensitivity to WNTs. Initially, it was thought that this phosphorylation results in a β-catenin stabilization and therefore promotes WNT/β-catenin signalling during the G2/M phase. Lately it was proposed that this event might be part of the WNT/STOP (Stabilization of proteins) signalling (Acebron et al., 2014). This pathway results in an inhibition of GSK-3β upon WNT stimulation, and since GSK-3β phosphorylates a plethora of proteins, which targets them for degradation, its inhibition results in a protein accumulation as a preparation for cell division. However, our and other reports suggest that apart from passive accumulation of proteins, the WNT signalling components have a functional and regulative role in the centrosomal biology.

Manipulating levels of DVL in the cell induces phenotypes, which could be connected to aberrant cell division or centrosomal function. SiRNA for DVL decreases the proliferation of cells and we assume this is caused by the failure of centrosomes to separate. On the other hand, the overexpression of DVL induces

formation of multinucleated cells. Such a phenotype is typically a consequence of an imbalance of centrosomal proteins including NEK2, Aurora A, PLK1, PLK4 and γ-tubulin (Tillement et al., 2009).

We also performed mass spectrometry analysis for the identification of novel DVL binding partners. Some of the identified proteins could be grouped according to their functions, thus suggesting a novel role of DVL in biological processes, such as DNA damage repair, spermatogenesis and centrosomal events. We decided to investigate the role of DVL in the centrosomal biology further.

First, we aimed to investigate the localization of endogenous DVL in cells. The overexpressed DVL creates distinct cytoplasmic puncta (Schwarz-Romond et al., 2007b), but a staining for endogenous DVL has been rather challenging. By utilizing low expression constructs, DVL was previously reported to be present at the spindle poles, the midbody (Kikuchi et al., 2010) and basal bodies (Park et al., 2008). We observed the DVL localization to the centrosome, which was mediated via the DIX domain, similar to what was reported for AXIN before (Alexandrova and Sokol, 2010). Additionally, several other proteins such as β-catenin (Bahmanyar et al., 2008; Huang et al., 2007) and kinases, such as CK1α and CK1ε were observed in the centrosome (Greer and Rubin, 2011; Milne et al., 2001; Stöter et al., 2005).

For several centrosomal proteins, which were identified by mass spectrometry as interaction partners of DVL, we confirmed their association to DVL by co-immunoprecipitation (co-IP). First, we investigated further DVL interaction with the centrosomal kinase NEK2. We found out that this interaction is mediated by the PDZ domain of DVL. We also analyzed the phosphorylation of DVL induced by NEK2 by mass spectrometry, which revealed many phosphorylation sites in DVL2 (27 sites) and DVL3 (41 sites), from which several are phosphorylated directly by NEK2, further suggesting that NEK2 regulates DVL activity. Meanwhile, the interaction of NEK2 and DVL was reported in functional screens in *Drosophila* (Schertel et al., 2013). In agreement with our findings, they also identified DVL as a substrate of NEK2 and the authors suggested that the interaction of DVL to NEK2 is dependent on its central part, which contains the PDZ domain (Schertel et al., 2013).

Next, we tested the interaction of DVL with other proteins of the centrosomal linker. A co-IP of C-NAP1 and CDK5RAP2 with DVL revealed a weak association, however, the interaction was strongly promoted by overexpression of NEK2. In agreement with this, we observed that an overexpression of DVL can displace these

proteins from the centrosome, a phenotype that was rescued by NEK2 knock-down. A similar mechanism was described earlier, where C-NAP1 was removed from the centrosome after the co-expression of NEK2 (Mayor et al., 2002). It seems that DVL serves as a scaffold protein in the loose centrosomal linker and undergoes a displacement from the centrosome upon NEK2 phosphorylation due to a change in DVL surface charge.

β-catenin is another component of WNT signalling, which is associated to the centrosomal linker. However, instead of an interaction with C-NAP1 and CDK5RAP2, it rather interacts with another linker protein, Rootletin (Bahmanyar et al., 2008). β-catenin is also phosphorylated by NEK2, but instead of degradation, it leads to its accumulation at the centrosome (Mbom et al., 2014) and β-catenin depletion increases the number of unseparated centrosomes, resulting in monopolar mitotic spindles (Kaplan et al., 2004). Another WNT signalling protein associated to the centrosome function is AXIN. Its loss leads to a centrosomal splitting (Hadjihannas et al., 2010).

A previous study reported that NEK2 serves as a strong enhancer of the WNT signalling pathway (Schertel et al., 2013). However, we were not able to observe any increase of WNT/β-catenin signalling via TOPFlash caused by overexpression of NEK2 itself, only in synergy with CK1. In contrast, removal of NEK2 by siRNA decreased WNT signalling. We hypothesized that NEK2-induced displacement of DVL from the centrosome increases the available pool of DVL in the cytoplasm. Thus, it is likely that NEK2 can positively influence WNT/β-catenin signalling indirectly by releasing more DVL, which can then signal in the WNT pathway. NEK2 can represent a novel regulatory mechanism influencing the intensity of WNT signalling and could connect it to both the centrosomal regulation and the cell cycle biology.

4.2 WNT signalling in angiogenesis of the CNS

(Paper II)

Many developmental and physiological processes require WNT signalling. One such processes is the development of the vasculature in CNS, where WNT signalling regulates sprouting of new blood vessels and establishment of BBB.

The requirement of WNT signalling for the initiation of the CNS angiogenesis was demonstrated in the brain endothelial cells (Daneman et al., 2009; Liebner et al., 2008). Various WNTs were detected in different areas of the developing CNS, with WNT-1, -3, -3A, -4 being expressed dorsally and WNT-7A and WNT-7B ventrally (Parr et al., 1993). Mice lacking WNT-7A/B are embryonically lethal at E12.5 because of a lethal disorganization of the neural tissue and an extensive haemorrhage (Stenman et al., 2008). The perineural vascular plexus is established normally, but it fails to reach the neural tissue, especially on the ventral side. Thus, WNT proteins serve as a potent migration signal in the CNS endothelial cells (Daneman et al., 2009).

A similar phenotype with defective vessel sprouting into the CNS was caused by depletion of β-catenin. Since β-catenin is one of the components in adherent junctions (Meng and Takeichi, 2009), it can be argued that its removal contributes to the haemorrhagic phenotype by destabilization of cell contacts (Cattelino et al., 2003). However, this does not seem to be the case, because the defects in the CNS vasculature (i) correspond only to the area where the WNT/β-catenin pathway is activated and (ii) are further caused by injecting the WNT/β-catenin pathway inhibitor affecting only β-catenin available for signalling but not influencing β-catenin in junctions (Daneman et al., 2009). Overall, we can conclude that WNT-7A and 7B are expressed by the neuronal cells to promote CNS vascularization by activating WNT/β-catenin signalling in endothelial cells.

A previous study indicated that endothelial cells of the CNS express FZDs mRNA (FZD_4, FZD_6 and FZD_{10}) at the onset of the angiogenesis (Hupe et al., 2017). FZD_4 has been previously connected to the retinal angiogenesis (Xu et al., 2004), where null mutation of FZD_4 or its ligand Norrin abrogated WNT signalling and caused the absence of intraretinal capillaries and defects in vascular patterning (Dejana, 2010; Ye et al., 2009). In the retina and the brain, FZD_4 helps to maintain the integrity of the BBB and the blood-retina barrier (BRB) (Wang et al., 2012). Additionally, both overactivation and loss of function of FZD_4 in mouse result in early embryonic lethality due to defects in vascular organization (Ye et al., 2009).

We decided to investigate the signalling aspects of FZD_{10}. The FZD_{10} mRNA is highly expressed in endothelial cells at the time of CNS angiogenesis. *In situ* hybridization experiments and immunostainings of FZD_{10} together with the typical endothelial marker, platelet endothelial cell adhesion molecule-1 (PECAM-1)

resulted in a colocalization in specific parts of the CNS of mouse embryo in E11.5 and E12.5. We showed that FZD_{10} forms the preassembly complex selectively with heterotrimeric G protein G13. Interestingly, G13 was shown to play a crucial role in the CNS angiogenesis. The G13 knock-out mice, as well as G13 endothelial specific knock out mice die *in utero* due to disorganized vasculature (Offermanns et al., 1997; Ruppel et al., 2005). However, the specifics of G13 downstream signalling in angiogenesis are not known.

Previously, it has been reported that one of the downstream signalling pathways of G13 can be YAP/TAZ signalling (Yu et al., 2012), known also as the Hippo pathway. This pathway controls the size of the organs and malfunction of the components of the Hippo pathway leads to a hippopotamus-like phenotype (Zhao et al., 2010). The YAP/TAZ signalling pathway represents an important regulator of angiogenesis by participating in regulation of endothelial cells proliferation (Shen and Stanger, 2015). Furthermore, overexpression of YAP enhances angiogenic sprouting and increases expression of angiopoietin 2, a molecule important for angiogenesis and vascular remodeling (Choi et al., 2015).

In addition to its role in angiogenesis in the CNS, WNT signalling plays a role in the establishment of the BBB. The expression of the BBB-specific protein GLUT-1 was induced by WNT-7A/B in endothelial cells (Daneman et al., 2009; Stenman et al., 2008). Additionally, activated WNT/β-catenin signalling repressed the immature brain vessel markers (Liebner et al., 2008). As mentioned previously, FZD_4 and Norrin signalling were also shown to be necessary for maintenance of the BBB (Wang et al., 2012). We showed that FZD_{10} colocalized with GLUT-1, the marker of the BBB. Therefore, we suggest that FZD_{10} might be involved in angiogenesis of the CNS and the establishment of BBB, most likely acting together with FZD_4. We showed that FZD_{10} forms a preassembly complex with G13 and that FZD_{10} activates the YAP/TAZ signalling pathway in a G13 dependent manner. The FZD_{10} activation of the YAP/TAZ pathway leading through G13 can be complementary to WNT/β-catenin signalling.

The WNT signalling might play a dual role in angiogenesis. WNT/β-catenin signalling can promote angiogenesis by cell proliferation. Further, it can be involved in the sprouting and migration of endothelial cells via G12/13 signalling and YAP/TAZ. Thus, the deregulation of both WNT/β-catenin-dependent

and -independent pathways could have distinct roles in the angiogenic process but result in similar angiogenic defects.

4.3 Role of the CRD and the linker domain

(Paper III)

Members of the Class FZD possess a large extracellular domain, the CRD, at their N-terminus. This bulky domain contains ten conserved cysteines engaging in five disulphide bonds and is seen as the main binding site for the WNT proteins (Bhanot et al., 1996; Povelones and Nusse, 2005) This has been structurally confirmed by the generation of the crystal structure of XWNT-8 bound to the FZD_8-CRD (Janda et al., 2012). However, the necessity of the CRD domain in WNT signalling has been challenged by several experiments *in vivo*. Phenotypes typical for defective β-catenin signalling in *Drosophila* embryos, where the genes for FZD homologues are mutated and cause disturbed segmentation of the cuticle, were rescued by FZDs lacking the CRD domain (Chen et al., 2004, 2008; Wu and Mlodzik, 2008). However, the PCP phenotype, which is represented by disrupted hair orientation in the *Drosophila* wing, was not rescued by the ΔCRD FZD (Wu and Mlodzik, 2008). One of the possible explanations is that the CRD is not necessary for WNT/β-catenin signalling but it is crucial for WNT/PCP signalling. The function of FZD in the PCP pathway might be different from β-catenin signalling and the transduction of the signal could be dependent on the interaction of the CRD with other components of the pathway.

Figure 12. A model of the structure, which represents FZD bound to the WNT-8 protein. The overlay model of FZD is based on the SMO structure (PDB: 5V57) and the FZD_8-CRD/WNT structure (PDB: 4F0A). WNT in red, CRD in yellow, linker domain in green and TM region in grey.

Nevertheless, the current understanding of CRD/WNT interaction suggests that the CRD serves as a high affinity binding platform for WNTs bringing them to the close proximity of the receptor, which leads to subsequent receptor activation. However, how the receptor translates the WNT binding into the activation of the TM core is still unknown. The WNT protein itself might be too large for being inserted or associated anywhere in the transmembrane domain (**Figure 12**). A better explanation could be extrapolated from the knowledge about SMO, where the binding of a small molecule ligand to the CRD causes a structural rearrangement in the transmembrane domain, which leads to receptor activation (Byrne et al., 2016). The recently solved structure of FZD_4 suggests that the extracellular part of the receptor, consisting of the linker domain and ECLs, is very compact and therefore such a 'communication' between the CRD and TMs might be possible (Yang et al., 2018). However, the CRD in the FZD_4 structure is missing, therefore the overall picture on the arrangement of the receptor's extracellular part might not completely reflect the reality.

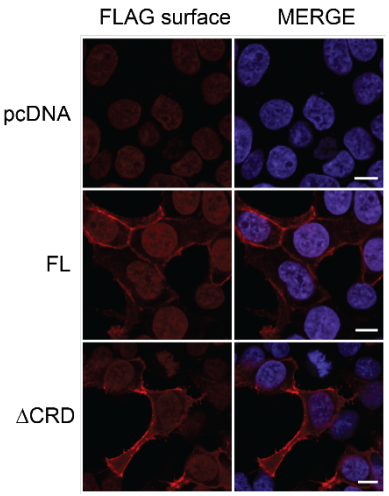

Figure 13. Surface expression of full-length and ΔCRD-FZD_6. The receptor is stained by FLAG antibody added into the media on the non-permeabilized cells.

This led us to investigate, whether the CRD is necessary for receptor activity, receptor embedding into the plasma membrane or if it exhibits any autoinhibitory functions. The function of the CRD was studied in SMO to greater extent, but the results are mostly contradictory. The deletion of the CRD resulted in constitutively active SMO (Byrne et al., 2016). However, this outcome was not supported by other studies, where the removal of SMO-CRD completely abolished the GLI reporter activity induced by SMO expression (Rana et al., 2013) (Aanstad et al., 2009) and *Drosophila* SMO mutants were not rescued by SMO lacking the CRD (Nakano et al., 2004).

To test the role of the CRD domain for FZD_6 functionality and signalling, we generated a construct of FZD_6, where the CRD domain was deleted (ΔCRD) but the construct retained the linker region, the TM core and the C-terminus. First, we

showed that such a deletion does not impair the ability of the receptor to be expressed at the cell surface (**Figure 13**). It is in agreement with the previous findings, where a similar truncation did not influence FZD insertion into the plasma membrane (Povelones and Nusse, 2005). Unfortunately, since FZD_6 is involved in the β-catenin-independent pathways, no robust assays for testing its functionality are available. As an indirect measure of activity, we used the common assays that rely on its intracellular interacting partner DVL. The DVL recruitment investigated by immunofluorescence revealed that both, full-length and ΔCRD are able to efficiently recruit DVL2. Similar results were obtained from the electrophoretic mobility shift of DVL. The DVL shift can be interpreted as a readout for DVL activation (Bernatik et al., 2011), and since DVL plays a role in both β-catenin-dependent and -independent branches, it is used as a common readout for the pathway activation. In agreement with the data from immunostaining, we observed DVL shift and hyper shift caused by both full length and ΔCRD. However, our data are in contrast to the previously published work, where full-length and ΔCRD FZD_6 constructs exhibited different behaviour (Golan et al., 2004). As a readout, the authors used TOPFlash reporter assay. FZD_6 cannot stimulate the WNT/β-catenin signalling pathway, but the authors reported that full-length but not ΔCRD FZD_6 repressed WNT/β-catenin signalling activated for example by WNTs or DVL overexpression. We repeated the experiment with overexpressed full-length and our version of ΔCRD FZD_6 and we observed similar decrease of WNT/β-catenin reporter activity for both constructs (**Figure 14**). Such a discrepancy in the results could be explained by specific location of the truncation in the protein sequence. As mentioned above, our construct possesses a substantial part of the linker domain. The constructs of FZD_6, which did not

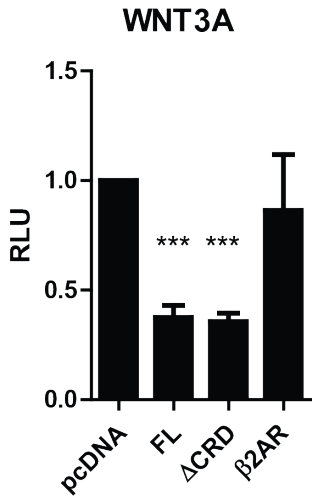

Figure 14. The effect of the FZD_6 expression on WNT/β-catenin signalling measured by TOPFlash luciferase reporter assay. FZD_6 reduces the agonist (WNT-3A) induced increase in the transcriptional activity irrespective of the presence of the CRD. The decrease of the signal is comparable for both, full-length and ΔCRD FZD_6 construct. The β_2 adreneric receptor (β_2AR) was used as a control.

exhibit functionality, were lacking the CRD, the linker domain and part of the TM core (Golan et al., 2004). The most likely explanation is that such a massive deletion impairs the insertion of the construct into the plasma membrane and therefore abolishing its function.

This argumentation is supported by the next set of experiments, where we determined a residue in the linker domain that is crucial for the insertion of FZD_6 into the plasma membrane. This residue in FZD_6 corresponds to the C161 and is fully conserved in both SMO and all FZDs. In the full-length SMO crystal structure, the cysteines in the protein linker are engaged in two disulphide bonds (Byrne et al., 2016). The C161 forms a disulphide bridge with C181 and C185 interacts with C260 of ECL1 (**Figure 15**). Interestingly, just simple substitution of any of the cysteines in the linker to alanine completely abolishes the insertion of the receptor into the cell surface, which is probably the reason for the receptor's inability to recruit DVL to the plasma membrane and to induce the DVL shift. Interestingly, from the immunoblots it is apparent that the FZD_6 mutants have impaired posttranslational modifications compared to the wild type receptor. The most plausible explanation is that the mutation of the cysteines in the linker disrupts the linker structure, which in turn prevents proper receptor folding and its subsequent insertion into the plasma membrane.

The importance of the cysteines is highlighted by the fact that corresponding cysteines in FZD_4 are found to be mutated in the patients with familial exudative vitreo-retinopathy (FEVR), a condition characterized by impaired vascularization in the retina in the eye. The mutation of these residues is linked to impaired receptor membrane localization or trafficking (Milhem et al., 2014), impaired ability to bind the ligand Norrin or impaired activation of the WNT/β-catenin pathway (Bang et al., 2018; Zhang et al., 2011). The importance of the linker domain in signalling and the ligand binding was recently confirmed: The CRD of FZD_4 together with the linker domain exhibited

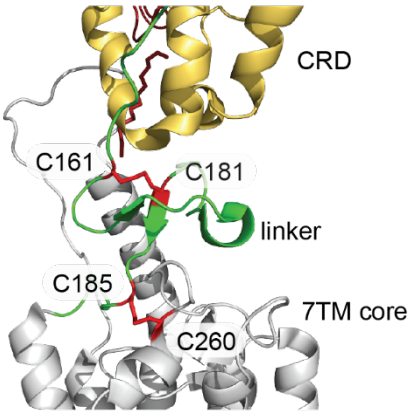

Figure 15. The model representing FZD_6, zoomed to the linker domain part (in green). The cysteines and the disulphide bonds in the linker domain are highlighted in red, the numbering corresponds to the numbering of FZD_6 (the model is based on the SMO structure, PDB: 5V57).

a ten times higher affinity to Norrin than CRD alone (Bang et al., 2018). Interestingly, swapping the linker domain from FZD_4 to linker from FZD_5 decreased Norrin binding and completely abolished Norrin-induced signalling.

Although previous research did not pay much attention to the linker domain, which was originally thought to be rather unstructured (DeBruine et al., 2017), it plays a crucial role is several processes. It is responsible for the receptor trafficking and insertion to the plasma membrane, as supported by our data in FZD_6 and reported by others in FZD_4 FEVR mutation, and it also participates in high-affinity ligand binding, receptor specificity and signal transduction (Bang et al., 2018). In addition, crystal structures of SMO indicate that the linker domain is tightly packed between the CRD and the TM core (Byrne et al., 2016) where it could function as a signal transducer.

4.4 Frizzleds as GPCRs

(Paper II and Paper IV)

The overall architecture of FZDs strongly suggests that they belong to the superfamily of GPCRs (Barnes et al., 1998). They possess conserved cysteines in the ECL1 and ECL2, which contribute to receptor stability by disulphide bridges. There are charged residues, in the ICL3, which could play role in G protein coupling. On the other hand, FZDs lack some domains conserved in Class A GPCRs, which are necessary for G protein coupling such as the DRY motif in TM3 or the NpxxxY in TM7 (Schulte, 2010).

From a functional point of view, the ability of FZDs to mediate signalling independently of heterotrimeric G protein in some experimental setups led to concerns about the GPCR nature of FZDs. Even more doubts arose after a recent finding that one homologue of FZDs, FZD_7, employs a non-receptor GEF protein, a DVL-associating protein with a high frequency of leucine residues called DAPLE, in order to activate G proteins (Aznar et al., 2015).

However, there is an overwhelming evidence for the involvement of heterotrimeric G proteins in both, β-catenin dependent and -independent signalling (Dijksterhuis et al., 2014; Schulte and Wright, 2018). One of the most frequent tools, which has been used to explore the heterotrimeric G protein engagement in WNT signalling pathways is a treatment by the *Bordetella pertussis* toxin (PTX), a toxin

inhibiting Gi, Go and Gt by ADP rybosylating the alpha subunit (Krueger and Barbieri, 1995).

One of the first experiments reporting an involvement of the heterotrimeric G protein in the β-catenin pathway was done in F9 teratocarcinoma stem cells. The activation of WNT/β-catenin signalling by WNT-5A and WNT-8 in FZD_1 expressing F9 cells causes their differentiation into primitive endoderm (Liu et al., 1999). Since this effect was blocked by PTX, it pointed to an involvement of G proteins from the Gi/o family. Similar experiments were performed with a chimeric receptor, composed of the intracellular parts of FZD and the transmembrane/extracellular domains from the β_2 adrenergic receptor (Liu et al., 2001). The researchers showed that the chimeric receptors can be stimulated with adrenergic ligands but would influence downstream signalling as FZDs. Work in *Drosophila melanogaster* also brought a piece of evidence for an involvement of G proteins in WNT signalling. Overexpression of Go increased the levels of WNT target genes in the *Drosophila* wing, (Katanaev et al., 2005). Our group also showed that stimulation of the mouse primary microglia cells with WNT-3A increased levels of β-catenin and phosphorylated LRP6 (Halleskog and Schulte, 2013). This effect can be diminished by PTX treatment suggesting an involvement of Gi/o.

Even more evidence emerged for the involvement of G proteins in β-catenin-independent pathways. In the *Drosophila* wing, the overexpression or depletion of Go resulted in impaired hair orientation in the wing, a phenotype typical for the PCP pathway (Katanaev and Tomlinson, 2006; Katanaev et al., 2005). Another role of G proteins in the PCP pathway was demonstrated in sensory organ precursor cells, where the Go was important for both correct positioning of the epithelial plane and intrinsic polarity (Gho and Schweisguth, 1998). The connection between the WNT/Calcium pathway and G proteins has also been discovered. Zebrafish embryos transfected with FZD_2, WNT-5A or both exhibited an increase in calcium mobilization (Slusarski et al., 1997b), which was inhibited by PTX, again suggesting the involvement of Gi/o.

In addition to the initial studies in cells and *in vivo*, the association of G proteins to FZDs were investigated by *in vitro* biochemical studies. One of the elegant approaches is to test whether FZD and G proteins can create an inactive 'preassembly' state, which is dissociated by addition of agonists. Such a preassembly state was shown for FZD_9 with Go using immunoprecipitation (Ramírez et al., 2016).

Our group successfully employed dcFRAP to investigate whether FZD_{10} can form a preassembly complex. We aimed to test at least one G protein from each subfamily (Gi1, Gq, Go, Gs, G12 and G13). In comparison to FZD_6, which associates with Gi and Gq (Kilander et al., 2014b), FZD_4 interacts with G12 and 13 (Arthofer et al., 2016), and FZD_5 with Gq (Wright et al., 2018). The preassembly state of FZD_{10} was specific just for G13 among all the tested G proteins. The receptor-G protein complex dissociated after addition of the endogenous agonists WNT-5A and -7A but not WNT-3A, highlighting the preference of FZD not only to specific heterotrimeric G protein but also to specific WNT in this readout. However, it is important to note that these experiments do not exclude interaction of the receptor with other G proteins via other mechanisms. Collision coupling can be one of such mechanisms, where the receptor and G protein interaction is based on their collision by free diffusion in the plasma membrane (Oldham and Hamm, 2008).

Furthermore, we were able to show the ability of FZD_{10} to signal through heterotrimeric G proteins by another technique – DMR, similarly as we presented for FZD_4 (Arthofer et al., 2016). The response to WNT-5A and WNT-7A was observed in wild type HEK293 cells only when FZD_{10} was overexpressed. The DMR response was lost in cells lacking G12/13, suggesting that FZD_{10} mediated the DMR response through G12/13. A treatment of non-transfected cells with WNT did not produce a consistent DMR despite an endogenous expression of most of the FZD homologues in HEK293 cells (Atwood et al., 2011). The possible explanation could be that endogenous levels of receptors signalling through G12/13 are not sufficient to observe a WNT-induced effect.

All of the abovementioned studies are cell-based, and therefore one can always argue for some intermediate cellular component mediating GEF function instead of the receptor. This intermediate player can reach sufficient amounts in the cells even without overexpression. We therefore decided to investigate the ability of FZD_6 to activate heterotrimeric G proteins in a reconstituted system, where the purified receptor is embedded into the well-defined phospholipid bilayer of a rHDL or nanodiscs. The rHDL particles are assembled *in vitro*, thus the composition of rHDL particle is exactly defined. We embedded FZD_6 into rHDL particles together with the purified heterotrimeric G protein, particularly Gi and Gq. These particles were used for a radioactive [^{35}S]GTPγS assay to quantify receptor-mediated activation of the G protein. Particles containing both heterotrimeric G protein and

FZD exhibited an elevated activation of G proteins in comparison to samples, where only G protein was added. The comparison of the activation of Gq and Gi shows a larger increase of the signal for Gi, which is known to have a higher rate of basal guanine nucleotide exchange than the other G proteins (Milligan, 2003). The elevated activation confirms that FZD acts as GEF and can activate the G protein directly. The observed increase is caused by the intrinsic GEF activity of the receptor, so-called constitutive activity. Our previous data suggests that monomeric receptor exhibits higher constitutive activity even in the absence of a ligand (Petersen et al., 2017).

Due to the technical problems, we were not able to observe a WNT induced increase in G protein activation on [^{35}S]GTPγS and these experiments need to be repeated. It is possible, that usage of WNTs in this assay will be challenging, as is the case for a WNT binding assay. We tried to reconstitute the WNT protein together with FZD and G protein, but even this setup did not result in increased G protein activation. In case that the technical challenges with WNT treatment remains, they could be overcome via creation of a chimeric receptor, where the WNT protein is covalently bound to FZD by replacing the CRD. Such a replacement was reported to produce a highly constitutively active receptor for WNT/β-catenin signalling (Povelones and Nusse, 2005). The WNT-FZD chimeric receptor could in our system bypass the necessity of usage of expensive WNTs and can stabilize the active state of the receptor. However, the mechanism of agonist binding including measuring the affinity cannot be studied in this setup.

How the interaction between the G protein and FZD is mediated within the HDL particles can be explained in several ways: (i) during the reconstitution, the heterotrimeric G protein itself is not efficiently reconstituted to the particle without the receptor and can fall off, in comparison to the sample where FZD is present and the G protein is able to directly associate to the receptor; (ii) the G protein is embedded into the particle in both cases, but the coupling and subsequent activation of the G protein can occur only in the sample with the receptor. In any case, this experiment provides the proof that FZD can bind and activate a heterotrimeric G protein in a reconstituted system, excluding participation of any intermediate players.

A complementary result to our finding was published previously, employing a bacterial system that naturally lacks the GPCR signalling machinery (Katanaev and Buestorf, 2009). In experiments, which aimed to prove that FZDs are functioning as

GPCR *per se*, the commercially produced G protein was added to the bacterial membranes with overexpressed human FZD. WNT stimulation induced G protein activation and addition of sFRP, proteins sequestering the WNTs thus functioning as negative regulators, inhibited the G protein activation.

Although FZDs can signal independently of heterotrimeric G proteins, we showed that they are able to function as a GPCR. Assembly of the inactive G protein signalling state cannot argue for the direct interaction but highlights that FZD-dependent signalling events include a heterotrimeric G protein. However, we showed that FZD can directly trigger G protein activation in a unique system of reconstituted phospholipid bilayer particles. Further optimization of this assay will hopefully lead to its application for screening of FZD modulators.

5 GENERAL DISCUSSION

The WNT signalling pathway is implicated in many pathophysiological processes and is therefore seen as a promising target for pharmaceutical intervention. The research presented in this thesis focuses on the less investigated functions of WNT signalling, with two main signalling molecules of the WNT signalling pathway in focus – the cytoplasmic protein DVL and the membrane receptor FZD. We further extend the knowledge about the role of DVL for the functionality and regulation of the centrosome and the centrosomal cycle. Moreover, we investigated the ability of FZDs to signal through and directly activate heterotrimeric G proteins and we also elucidated the role of the poorly described linker region of FZD connecting the extracellular CRD domain and the receptor core.

The connection of WNT signalling to the centrosome and the centrosomal cycle has been emerging over the last years. The general notion appeared after the finding that many of the key players of the WNT signalling pathway localize to the centrosome or centrosome-associated structures, such as the basal bodies (Bryja et al., 2017). However, a fundamental understanding of the underlying mechanisms of WNT signalling during various centrosomal events is still missing, leaving many questions unanswered. For example, is WNT signalling regulating the centrosomal events or *vice versa*, do centrosomal events influence the intensity or the efficacy of WNT signalling? Is it just a coincidence that many WNT signalling proteins are localized at the centrosome? Do they have a specific function in the centrosome? Results in this thesis do not answer all these questions but contribute to elucidate a part of the complex WNT signalling mosaic.

We have shown that DVL plays a role in the centrosome separation as a part of the loose centrosomal linker. Interestingly, other proteins involved in WNT signalling, such as β-catenin and AXIN, were also shown to participate in the centrosomal separation (Kim et al., 2009; Mbom et al., 2014). This naturally raises the question, whether the role of WNT signalling is instructive, or whether the individual proteins act alone and not in a coordinated manner. Do WNT signalling proteins exist in distinct non-overlapping pools (at the centrosome, in the cytoplasm or at the membrane), or is there a bustling communication going on? If there is indeed some communication, does it have an effect on the outcomes of WNT signalling such as level of activation or even WNT/β-catenin-dependent and independent pathway switching? We have shown that the release of DVL from the centrosome might increase its

availability for functioning in the β-catenin-dependent WNT signalling. However, if the centrosomal cycle is more globally connected to WNT signalling outcome remains to be described.

Part of the study closely investigated the main receptor for WNTs, FZD. The knowledge of how FZDs function is still one of the biggest mysteries in WNT signalling. The research is complicated by several facts, most relevant being the lack of assays detecting the direct activation of FZDs or ligand-FZD binding. Until now, the assays most commonly monitor events downstream of receptor activation but suffer from possible signal amplification or suppression by intermediate players. Any of the co-receptors, kinase cascades, second messengers or scaffold proteins can change the signalling output. The long-term aim of our laboratory is to develop assays monitoring the direct activation of FZDs. In addition to the presented [^{35}S]GTPγS assay utilizing the rHDL particles with the embedded receptor, we have constructed Förster resonance energy transfer (FRET) probes for another FZD homologue, FZD_5. The probes reflect conformational changes upon receptor stimulation and present the first direct measurement of WNT-induced structural changes of FZD (Wright et al., 2018). This methodology presents a direct consequence of a ligand binding in form of a conformational change and is thereby complementary to a putative ligand binding assay and the [^{35}S]GTPγS assay presented in the paper IV and [^{35}S]GTPγS assay performed on cell membranes (Kilander et al., 2011a, 2011b; Koval and Katanaev, 2011).

The development of assays for monitoring direct receptor activation greatly contributes not only to understanding of the receptor activation mechanism, but it also helps to overcome another obstacle: it enables the screening of ligands suitable for biochemical and pharmacological assays. WNT proteins that serve as FZD ligands are lipidated and their production, purification and usage are expensive and challenging as a result of the lipid moieties. However, a reliable assay monitoring the direct receptor activation is one of the prerequisites for small molecule or other ligand screening. It is very difficult to exit this vicious circle. The situation is even more complicated by the many possible interactions between 19 WNTs and ten FZDs, which are only partially elucidated. However, it is clear that specific WNTs can signal through multiple FZDs and similarly one FZD can be activated by multiple WNTs (Dijksterhuis et al., 2015). It should also be noted that there is a degree of functional redundancy among the FZD homologues. Unfortunately, cell lines and tissues usually express multiple FZDs and therefore it is difficult to dissect their individual contributions.

Our main finding of the work presented in this thesis support the evidence that FZDs behave as classical GPCRs, in other words that they can directly activate heterotrimeric G proteins. The doubts about their GPCR nature arose due to their ability to signal independently of heterotrimeric G proteins as well as previously mentioned obstacles. We demonstrated in a completely isolated system of rHDL particles that FZD_6 can activate G proteins with its intrinsic constitutive activity. Developing an assay to directly monitor the signalling outcome of FZD can greatly improve our understanding of FZDs and can contribute to the development of new FZD modulators. Moreover, we showed that embedding functional FZDs into rHDL particles is possible. Apart from the use of rHDL particles in biochemical and pharmacological assays, they might enable us to elucidate the full-length structure of FZD. FZDs embedded in rHDL particles could be visualized by Cryo EM, a technique becoming more popular for proteins that resist crystallization. Although FZD_4 has been recently crystallized, the assessment of the full-length structure via Cryo EM requires smaller amounts of protein and could provide a possibility to visualize FZDs in a complex for example with a heterotrimeric G protein or WNT proteins.

During the optimization of the purification procedure, we noticed the presence of several truncation products. We do not know whether they are physiologically relevant, however, we decided to test their functionality. We designed and created several truncation products and found out that the linker region in FZD_6 was critical for receptor presence in the plasma membrane. Surprisingly, the corresponding cysteine residues in this region, which are responsible for the presence of the receptor in the plasma membrane, are found to be mutated in the FZD_4 homologue, causing an impaired retinal development. The recent finding that the linker region increases Norrin affinity to FZD_4 and determines their specificity (Bang et al., 2018) raises the interesting idea that in case of FZDs, the ligand specificity is not determined by the CRD but by the linker region.

To conclude, the improved understanding of the signal transduction and regulation of the WNT signalling pathway will shed light on basic mechanisms in development and disease progression. Moreover, understanding the molecular details of receptor activation and dynamics allows the development of small molecule drugs targeting FZDs with more precision with regard to the receptor-mediated signal initiation, pathway selectivity and less side effects.

6 CONCLUSIONS

- DVL is a part of the centrosomal linker and regulates centrosomal separation
- FZD_{10} establishes a preassembly complex with heterotrimeric G13
- The downstream signalling pathways of FZD_{10} involve G13, YAP/TAZ and can potentially be involved in angiogenesis of the CNS
- The cysteines in the linker region of FZD_6 are necessary for receptor embedding into the plasma membrane and for its proper functionality
- DVL and the isolated DEP domain interact with FZD_6 independently of agonist stimulation
- Embedding FZD_6 into rHDL particles allows assessment of their GEF activity using the [^{35}S]GTPγS assay
- Monomeric and purified FZD_6 is able to activate heterotrimeric G proteins, particularly Gi1, with its constitutive activity

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